

*Application  
for  
United States Letters Patent*

**To all whom it may concern:**

*Be it known that*

**Neil T. Parkin and Rainer A. Ziermann**

*have invented certain new and useful improvements in*

**MEANS AND METHODS FOR MONITORING PROTEASE INHIBITOR ANTIRETROVIRAL  
THERAPY AND GUIDING THERAPEUTIC DECISIONS IN THE TREATMENT OF HIV/AIDS**

*of which the following is a full, clear and exact description.*

**MEANS AND METHODS FOR MONITORING PROTEASE  
INHIBITOR ANTIRETROVIRAL THERAPY AND GUIDING  
THERAPEUTIC DECISIONS IN THE TREATMENT OF HIV/AIDS**

This application claims the benefit of U.S. Application No. 09/766,344, filed January 19, 2001 the contents of which are hereby incorporated by reference into this application.

5 Throughout this application, various references are referred to within parenthesis. Disclosures of these publications in their entirety are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains.

10 **Technical Field**

This invention relates to antiretroviral drug susceptibility and resistance tests to be used in identifying effective drug regimens for the treatment of human immunodeficiency virus (HIV) infection and acquired immunodeficiency syndrome (AIDS). The invention further relates to the means and methods of monitoring the clinical progression of HIV infection and its response to antiretroviral therapy using phenotypic or genotypic susceptibility assays. The invention also relates to novel vectors, host cells and compositions for carrying out phenotypic susceptibility tests. The invention further relates to the use of various genotypic methodologies to identify patients who do not respond to a particular antiretroviral drug regimen. This invention also relates to the screening of candidate antiretroviral drugs for their capacity to inhibit viral replication,

selected viral sequences and/or viral proteins. More particularly, this invention relates to the determination of protease inhibitor (PRI) susceptibility using phenotypic or genotypic susceptibility tests. This invention also relates to a means and method for accurately and reproducibly measuring viral replication fitness.

#### Background of the Invention

10 HIV infection is characterized by high rates of viral turnover throughout the disease process, eventually leading to CD4 depletion and disease progression. Wei X, Ghosh SK, Taylor ME, et al. (1995) Nature 343, 117-122 and Ho DD, Naumann AU, Perelson AS, et al. (1995) Nature 373, 123-126. The aim of antiretroviral therapy is to achieve substantial and prolonged suppression of viral replication. Achieving sustained viral control is likely to involve the use of sequential therapies, generally each therapy comprising combinations of three or more antiretroviral drugs. Choice of initial and subsequent therapy should, therefore, be made on a rational basis, with knowledge of resistance and cross-resistance patterns being vital to guiding those decisions. The primary rationale of combination therapy relates to synergistic or additive activity to achieve greater inhibition of viral replication. The tolerability of drug regimens will remain critical, however, as therapy will need to be maintained over many years.

30 In an untreated patient, some  $10^{10}$  new viral particles are produced per day. Coupled with the failure of HIV reverse transcriptase (RT) to correct transcription errors by exonucleolytic proofreading, this high level of viral

turnover results in  $10^4$  to  $10^5$  mutations per day at each position in the HIV genome. The result is the rapid establishment of extensive genotypic variation. While some template positions or base pair substitutions may be more error prone (Mansky LM, Temin HM (1995) J Virol 69, 5087-5094) (Schinazi RF, Lloyd RM, Ramanathan CS, et al. (1994) Antimicrob Agents Chemother 38, 268-274), mathematical modeling suggests that, at every possible single point, mutation may occur up to 10,000 times per day in infected individuals.

For antiretroviral drug resistance to occur, the target enzyme must be modified while preserving its function in the presence of the inhibitor. Point mutations leading to an amino acid substitution may result in changes in shape, size or charge of the active site, substrate binding site or in positions surrounding the active site of the enzyme. Mutants resistant to antiretroviral agents have been detected at low levels before the initiation of therapy. (Mohri H, Singh MK, Ching WTW, et al. (1993) Proc Natl Acad Sci USA 90, 25-29) (Nájera I, Richman DD, Olivares I, et al. (1994) AIDS Res Hum Retroviruses 10, 1479-1488) (Nájera I, Holguin A, Quiñones-Mateu E, et al. (1995) J Virol 69, 23-31). However, these mutant strains represent only a small proportion of the total viral load and may have a replication or competitive disadvantage compared with wild-type virus. (Coffin JM (1995) Science 267, 483-489). The selective pressure of antiretroviral therapy provides these drug-resistant mutants with a competitive advantage and thus they come to represent the dominant quasi species (Frost SDW, McLean AR (1994) AIDS 8, 323-332) (Kellam P, Boucher CAB, Tijnagal JMGH (1994) J



Gen Virol 75, 341-351) ultimately leading to a rebound in viral load in the patient.

5 Early development of antiretroviral therapy focused on inhibitors of reverse transcriptase. Both nucleoside and non-nucleoside inhibitors of this enzyme showed significant antiviral activity (DeClerq, E. (1992) AIDS Res. Hum. Retrovir. 8:119-134). However, the clinical benefit of these drugs had been limited due to drug resistance, limited potency, and host cellular factors (Richman, D.D. (1993) Ann. Rev. Pharm. Tox. 32:149-164). Thus inhibitors targeted against a second essential enzyme of HIV were urgently needed.

15 In 1988, the protease enzyme of HIV was crystallized and its three-dimensional structure was determined, (Navia MA, Fitzgerald PMD, McKeever BM, Leu CT, Heimbach JC, Herber WK, Sigal IS, Darke PL, Springer JP (1989) Nature 337:615-620 and Winters MA, Schapiro JM, Lawrence J, Merigan TC (1997) In Abstracts of the International Workshop on HIV Drug Resistance, Treatment Strategies and Eradication, St. Petersburg, Fla.) allowing for the rapid development of protease inhibitors. Initially, it was hypothesized that HIV protease, unlike reverse transcriptase, would be unable to accommodate mutations leading to drug resistance. This is not the case, and to date over 20 amino acid substitutions in the HIV protease have been observed during treatment with the currently available protease inhibitors. The genetic pattern of mutations conferring resistance to these protease inhibitors is complex, and cross-resistance between structurally different compounds occurs.

PROTEASE INHIBITORS

HIV protease was classified as an aspartic proteinase on the basis of putative active-site homology (Toh H, Ono M, Saigo K, Miyata T (1985) Nature 315:691), its inhibition by peptastin (Richards AD, Roberts R, Dunn BM, Graves MC, Kay J (1989) FEBS Lett 247:113), and its crystal structure (Navia MA, Fitzgerald PMD, McKeever BM, Lau CT, Heimbach JC, Herber WK, Sigal IS, Darke PL, Springer JP (1989) Nature 337:615-620). The enzyme functions as a homodimer composed of two identical 99-amino acid chains (Debouck C, Navia MA, Fitzgerald PMD, McKeever BM, Lau CT, Heimbach JC, Herber WK, Sigal IS, Darke PL, Springer JP (1988) Proc. Natl. Acad. Sci. USA 84:8903-8906), with each chain containing the characteristic Asp-Thr-Gly active-site sequence at positions 25 to 27 (Toh H, Ono M, Saigo K, Miyata T (1985) Nature 315:691).

HIV protease processes gag (p55) and gag-pol (p160) polyprotein products into functional core proteins and viral enzymes (Kohl NE, Diehl RE, Rands E, Davis LJ, Hanobik MG, Wolanski B, Dixon RA (1991) J. Virol. 65:3007-3014 and Kramer RA, Schaber MD, Skalka AM, Ganguly K, Wong-Staal F, Reddy EP (1986) Science 231:1580-1584). During or immediately after budding, the polyproteins are cleaved by the enzyme at nine different cleavage sites to yield the structural proteins (p17, p24, p7, and p6) as well as the viral enzymes reverse transcriptase, integrase, and protease (Pettit SC, Michael SF, Swanstrom R (1993) Drug Discov. Des. 1:69-83).

An asparagine replacement for aspartic acid at active-site residue 25 results in the production of noninfectious

5 viral particles with immature, defective cores (Huff JR  
(1991) AIDS J. Med. Chem. 34:2305-2314, Kaplan AH, Zack  
JA, Knigge M, Paul DA, Kempf DJ, Norbeck DW, Swanstrom R  
(1993) J. Virol. 67:4050-4055, Kohl NE, Emini EA, Schleif  
10 WA, Davis LJ, Heimbach JC, Dixon RA, Scolnik EM, Sigal IS  
(1988) Proc. Natl. Acad. Sci. USA 85:4686-4690, Peng C, Ho  
BK, Chang TW, Chang NT (1989) J. Virol. 63:2550-2556).  
Similarly, wild-type virus particles produced by infected  
cells treated with protease inhibitors contain unprocessed  
15 precursors and are noninfectious (Crawford S, Goff SP  
(1985) J. Virol. 53:899-907, Gottlinger HG, Sodroski JG,  
Haseltine WA (1989) Proc. Natl. Acad. Sci. USA  
86:5781-5785, Katoh IY, Yoshinaka Y, Rein A, Shibuya M,  
Odaka T, Oroszlan S (1985) Virology 145:280-292, Kohl NE,  
20 Emini EA, Schleif WA, Davis LJ, Heimbach JC, Dixon RA,  
Scolnik EM, Sigal IS (1988) Proc. Natl. Acad. Sci. USA  
85:4686-4690, Peng C, Ho BK, Chang TW, Chang NT (1989) J.  
Virol. 63:2550-2556, Stewart L, Schatz G, Wogt VM (1990)  
J. Virol. 64:5076-5092). Unlike reverse transcriptase  
25 inhibitors, protease inhibitors block the production of  
infectious virus from chronically infected cells (Lambert  
DM, Petteway, Jr. SR, McDanal CE, Hart TK, Leary JJ,  
Dreyer GB, Meek TD, Bugelski PJ, Bolognesi DP, Metcalf BW,  
Matthews TJ (1992) Antibicrob. Agents Chemother.  
30 36:982-988). Although the viral protease is a symmetric  
dimer, it binds its natural substrates or inhibitors  
asymmetrically (Dreyer, GB, Boehm JC, Chenere B,  
DesJarlais RL, Hassell AM, Meek TD, Tomaszek TAJ, Lewis M  
(1993) Biochemistry 32:937-947, Miller MJ, Schneider J,  
Sathyanarayana BK, Toth MV, Marshall GR, Clawson L, Selk  
L, Kent SB, Wlodawer A (1989) Science 246:1149-1152).  
These findings together with the knowledge that amide

bonds of proline residues are not susceptible to cleavage by mammalian endopeptidases gave rise to the first class of HIV-1 protease inhibitors based on the transition state mimetic concept, with the phenylalanine-proline cleavage site being the critical nonscissile bond (Roberts NA, Martin JA, Kinchington D, Broadhurst AV, Craig JC, Duncan IB, Galpin SA, Handa BK, Kay J, Krohn A, Lambert RW, Merett JH, Mills JS, Parkes KEB, Redshaw S, Ritchie AJ, Taylor DL, Thomas GJ, Machin PJ (1990) Science 248:358-361).

**Amino acids implicated in resistance to protease inhibitors.**

As new protease inhibitors are developed, the ability of certain amino acid substitutions to confer resistance to the inhibitor is usually determined by several methods, including selection of resistant strains in vitro, site-directed mutagenesis, and determination of amino acid changes that are selected during early phase clinical trials in infected patients. While some amino acid substitutions are specifically correlated with resistance to certain protease inhibitors (see below), there is considerable overlap between sets of mutations implicated in resistance to all approved protease inhibitors. Many investigators have attempted to classify these mutations as either being "primary" or "secondary", with varying definitions. For example, some investigators classify as primary mutations which are predicted, based on X-ray crystallographic data, to be in the enzyme active site with the potential for direct contact with the inhibitor (e.g. D30N, G48V, I50V, V82A/F/S/T, I84V, N88S, L90M).

Secondary mutations are usually considered as being compensatory for defects in enzyme activity imposed by primary mutations, or as having enhancing effects on the magnitude of resistance imparted by the primary mutations (e.g. L10I/F/R/V, K20I/M/R/T, L24I, V32I, L33F/V, M36I/L/V, M46I/L/V, I47V, I54L/V, L63X, A71T/V, G73A/S/T, V77I, N88D). Lists of mutations and corresponding inhibitors are maintained by several organizations, for example: Schinazi et al., Mutations in retroviral genes associated with drug resistance, *Intl. Antiviral News* 1999,7:46-69 and Shafer et al., Human Immunodeficiency Virus Reverse Transcriptase and Protease Sequence Database, *Nucleic Acids Research* 1999, 27(1), 348-352 (also accessible via the internet at <http://www.viral-resistance.com/> or <http://hivdb.stanford.edu/hiv/>)

Saquinavir

Saquinavir, developed by Hoffmann-La Roche, was the first protease inhibitor to undergo clinical evaluation, demonstrating that HIV-1 protease was a valid target for the treatment of HIV infection (Jacobsen H, Brun-Vezinet F, Duncan I, Hanggi M, Ott M, Vella S, Weber J, Mous J (1994) J. Virol. 68:2016-2020). Saquinavir is a highly active peptidomimetic protease inhibitor with a 90% inhibitory concentration (IC90) of 6 nM (id). In vitro, saquinavir can select for variants with one or both of two amino acid substitutions in the HIV-1 protease gene, a valine-for-glycine substitution at position 48 (G48V), a methionine-for-leucine substitution at residue 90 (L90M), and the double substitution G48V-L90M (Eberle J, Bechowsky B, Rose D, Hauser U, vonder Helm K, Guertler L, Nitschko H (1995) AIDS Res. Hum. Retroviruses 11:671-676, Jacobsen H, Yasargil K, Winslow DL, Craig JC, Kroehn A, Duncan IB, Mous J (1995) Virology 206:527-534, Turriziani O, Antonelli G, Jacobsen H, Mous J, Riva E, Pistello M, Dianzani F (1994) Acta Virol. 38:297-298). In most cases, G48V is the first mutation to appear, and continued selection results in highly resistant double-mutant variants. A substitution at either residue results in a 3- to 10-fold decreased susceptibility to the inhibitor, whereas the simultaneous occurrence of both substitutions causes a more severe loss of susceptibility of >100-fold (id).

In vivo, saquinavir therapy appears to select almost exclusively for mutations at codons 90 and 48 (id, Jacobsen H, Hangi M, Ott M, Duncan IB, Owen S, Andreoni M, Vella S, Mous J (1996) J. Infect. Dis. 173:1379-1387, Vella S, Galluzzo C, Giannini G, Pirillo MF, Duncan I,

Jacobsen H, Andreoni M, Sarmati L, Ercoli L (1996) Antiviral Res. 29:91-93). Saquinavir-resistant variants emerge in approximately 45% of patients after 1 year of monotherapy with 1,800 mg daily (Craig IC, Duncan IB, Roberts NA, Whittaker L (1993) In Abstracts of the 9th International Conference on AIDS, Berlin, Germany, Duncan IB, Jacobsen H, Owen S, Roberts NA (1996) In Abstracts of the 3rd Conference of Retroviruses and Opportunistic Infections, Washington, D.D., id, Mous J, Brun-Vezinet F, Duncan IB, Haenggi M, Jacobsen H, Vella S (1994) In Abstracts of the 10th International Conference on AIDS, Yokohama, Japan). The frequency of resistance is lower (22%) in patients receiving combination therapy with zidovudine, zalcitabine, and saquinavir (Collier AC, Coombs R, Schoenfeld DA, Bassett RL, Joseph Timpone MS, Baruch A, Jones M, Facey K, Whitacre C, McAuliffe VJ, Friedman HM, Merigan TC, Reichmann RC, Hooper C, Corey L (1996) N. Engl. J. Med. 334:1011-1017). In contrast to in vitro-selected virus, where the G48V mutation is the first step to resistance, the L90M exchange is the predominant mutation selected in vivo while the G48V (2%) or the double mutant (<2%) is rarely found (id). In another recent study of in vivo resistance during saquinavir monotherapy no patient was found to harbor a G48V mutant virus (Ives KJ, Jacobsen H, Galpin SA, Garaev MM, Dorrell L, Mous J, Bragman K, Weber JN (1997 J. Antimicrob. Chemother. 39:771-779). Interestingly, Winters et al. (id) observed a higher frequency of the G48V mutation in patients receiving higher saquinavir doses as monotherapy. All patients (six of six) who initially developed G48V also acquired a V82A mutation either during saquinavir treatment or after switching to either indinavir or



nefinavir. An identical mutational pattern was found in another study during saquinavir monotherapy (Eastman PS, Duncan IB, Gee C, Race E (1997) In Abstracts of the International Workshop on HIV Drug Resistance, Treatment Strategies and Eradication, St. Petersburg, Fla.). Some residues represent sites of natural polymorphism of the HIV-1 protease (positions 10, 36, 63, and 71) and appear to be correlated to the L90M mutation (id). Another substitution, G73S, has been recently identified and may play a role in saquinavir resistance in vivo. Isolates from five patients with early saquinavir resistance and those from two patients with induced saquinavir resistance after a switch of therapy to indinavir carried the G73S and the L90M substitutions Dulioust A, Paulous S, Guillemot L, Boue F, Galanaud P, Clavel F (1997) In Abstracts of the International Workshop on HIV Drug Resistance, Treatment Strategies and Eradication, St. Petersburg, Fla.).

20 **Ritonavir**

Ritonavir, developed by Abbott Laboratories, was the second HIV protease inhibitor to be licensed in the United States. Ritonavir is a potent and selective inhibitor of HIV protease that is derived from a C2-symmetric, peptidomimetic inhibitor (Ho DD, Toyoshima T, Mo H, Kempf DJ, Norbeck D, Chen CM, Wideburg NE, Burt SK, Erickson JW, Singh MK (1994) J. Virol. 68:2016-2020). In vitro activity has been demonstrated against a variety of laboratory strains and clinical isolates of HIV-1 with IC90s of 70 to 200 nM (Kuroda MJ, El-Farrash MA, Clouthury S, Harada S (1995) Virology 210:212-216).



Resistant virus generated by serial in vitro passages is associated with specific mutations at positions 84, 82, 71, 63, and 46 (Markowitz M, Mo H, Kempf DJ, Norbeck DW, Bhat TN, Erickson JW, Ho DD (1995) J. Virol. 69:701-706).

5 The I84V substitution appeared to be the major determinant of resistance, resulting in a 10-fold reduction in sensitivity to ritonavir. Addition of the V82F mutation confers an even greater level of resistance, up to 20-fold. The substitutions M46I, L63P, and A71V, when  
10 introduced into the protease coding region of wild-type NL4-3, did not result in significant changes in drug susceptibility. Based on replication kinetics experiments, these changes are likely to be compensatory for active-site mutations, restoring the impaired  
15 replicative capacity of the combined V82F and I84V mutations.

104000-244450

Indinavir

Indinavir, developed by Merck & Co., is the third HIV protease inhibitor licensed in the United States. Indinavir is a potent and selective inhibitor of HIV-1 and HIV-2 proteases with  $K_i$  values of 0.34 and 3.3 nM, respectively (Vacca Jp, Dorsey BD, Schleif WA, Levin RB, McDaniel SL, Darke PL, Zugay J, Quintero JC, Blahy OM, Roth E, Sardana VV, Schlabach AJ, Graham PI, Condra JH, Gotlib L, Holloway MK, Lin J, Chen L-w, Vastag K, Ostobich D, Anderson PS, Emini EA, Huff JR (1994) Proc. Natl. Acad. Sci. USA 91:4096-4100). The drug acts as peptidomimetic transition state analogue and belongs to the class of protease inhibitors known as HAPA (hydroxyaminopentane amide) compounds (ibid). Indinavir provides enhanced aqueous solubility and oral bioavailability and in cell culture exhibits an  $IC_{95}$  of 50 to 100 nM (Emini EA, Schleif WA, Deutsch P, Condra JH (1996) Antiviral Chemother. 4:327-331).

Despite early reports of a lack of in vitro resistance by selection with indinavir (id), Tisdale et al. (Tisdale M, Myers RE, Maschera B, Parry NR, Oliver NM, Blair ED (1995) Antibicrob. Agents Chemother. 39:1704-1710) were able to obtain resistant variants during selection in MT-4 cells with substitutions at residues 32, 46, 71, and 82. At least four mutations were required to produce a significant loss of susceptibility (6.1-fold compared with the wild type). The mutation at position 71, described as compensatory (Markowitz M, Mo H, Kempf DJ, Norbeck DW, Bhat TN, Erickson JW, Ho DD (1995) J. Virol. (id), appeared to contribute phenotypic resistance and also to improve virus growth. Emini et al. (id) and Condra et al.

(Condra JH, Holder DJ, Schleif WA, Blahy OM, Danovich RM, Gabryelski LJ, Graham DJ, Laird D, Quintero JC, Rhodes A, Robbins HL, Roth E, Shivaprakash M, Yang T, Chodakewitz JA, Deutsch PJ, Leavitt RY, Massari Fe, Mellors JW, Squires KE, Steigbigel RT, Teppler H, Emini EA (1995) Nature 374:569-571) found by constructing mutant HIV-1 clones that at least three mutations at residues 46, 63, and 82 were required for the phenotypic manifestation of resistance with a fourfold loss of susceptibility.

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101090-224260

**Nelfinavir**

Nelfinavir, developed by Agouron Pharmaceuticals, is a selective, nonpeptidic HIV-1 protease inhibitor that was designed by protein structure-based techniques using iterative protein crystallographic analysis (Appelt KR, Bacquet J, Bartlett C, Booth CLJ, Freer ST, Fuhry MM, Gehring MR, Herrmann SM, Howland EF, Janson CA, Jones TR, Kan CC, Kathardekar V, Lewis KK, Marzoni GP, Mathews DA, Mohr C, Moomaw EW, Morse CA, Oatley SJ, Ogden RC, Reddy MR, Reich SH, Schoettlin WS, Smith WW, Varney MD, Villafranca JE, Ward RW, Webber S, Webber SE, Welsh KM, White J (1991) J. Med. Chem. 34:1925-1928). In vitro, nelfinavir was found to be a potent inhibitor of HIV-1 protease with a  $K_i$  of 2.0 nM (Kaldor SW, Kalish VJ, Davies JF, Shetty BV, Fritz JE, Appelt K, Burgess JA, Campanale KM, Chirgadze NY, Clawson DK, Dressman BA, Hatch SD, Khalil DA, Kosa MB, Lubbehusen PP, Muesing MA, Patrick AK, Reich SH, Su KS, Tatlock JH (1997) J. Med. Chem. 40:3979-3985). The drug demonstrated antiviral activity against several laboratory and clinical HIV-1 and HIV-2 strains with 50% effective concentrations ranging from 9 to 60 nM (Patick AK, Boritzki TJ, Bloom LA (1997) Antimicrob. Agents Chemother. 41:2159-2164). Nelfinavir exhibits additive-to-synergistic effects when combined with other antiretroviral drugs (Partaledis JA, Yamaguchi AK, Tisdale M, Blair EE, Falcione C, Maschera B, Myers RE, Pazhanisamy S, Futer O, Bullinan AB, Stuver CM, Byrn RA, Livingston DJ (1995) J. Virol. 69:5228-5235). Preclinical data showed high levels of the drug in mesenteric lymph nodes and the spleen and good oral bioavailability (Shetty BV, Kosa MB, Khalil DA, Webber S (1996) Antimicrob. Agents Chemother. 40:110-114).

In vitro, following 22 serial passages of HIV-1<sub>NL4-3</sub> in the presence of nelfinavir, a variant (P22) with a sevenfold reduced susceptibility was isolated. After an additional six passages a variant (P28) with a 30-fold-decreased susceptibility to nelfinavir was identified (Patick AK, Ho H, Markowitz M, Appelt K, Wu B, Musick L, Kaldor S, Reich S, Ho D, Webber S (1996) Antimicrob. Agents Chemother. 40:292-297). Sequence analysis of the protease gene from these variants identified in decreasing frequency the substitutions D30N, A71V, and I84V for the P22 variant and mutations M46I, I84V/A, L63P, and A71V for the P28 variant. Antiviral susceptibility testing of recombinant mutant HIV-1<sub>NL4-3</sub> containing various mutations resulted in a fivefold-increased 90% effective concentration for the I84V and D30N single mutants and the M46I/I84V double mutant, whereas no change in susceptibility was observed with M46I, L63P, or A71V alone (*ibid*).

Amprenavir

Amprenavir is a novel protease inhibitor developed by Vertex Laboratories and designed from knowledge of the HIV-1 protease crystal structure (Kim EE, Baker CT, Dyer MD, Murcko MA, Rao BG, Tung RD, Navia MA (1995) J. Am. Chem. Soc. 117:1181-1182). The drug belongs to the class of sulfonamide protease inhibitors and has been shown to be a potent inhibitor of HIV-1 and HIV-2, with IC50s of 80 and 340 nM, respectively. The mean IC50 for amprenavir against clinical viral isolates was 12 nM (St. Clair MH, Millard J, Rooney J, Tisdale M, Parry N, Sadler BM, Blum MR, Painter G (1996) Antiviral Res. 29:53-56). HIV-1 variants 100-fold resistant to amprenavir have been selected by in vitro passage experiments (id). DNA sequence analysis of the protease of these variants revealed a sequential accumulation of point mutations resulting in amino acid substitutions L10F, M46I, I47V, and I50V. The key resistance mutation in the HIV-1 protease substrate binding site is I50V. As a single mutation it confers a two- to threefold decrease in susceptibility (ibid). The other substitutions did not result in reduced susceptibility when introduced as single mutations into an HIV-1 infectious clone (HXB2). However, a triple protease mutant clone containing the mutations M46I, I47V, and I50V was 20-fold less susceptible to amprenavir than wild-type virus. The I50V mutation has not been frequently reported in resistance studies with other HIV protease inhibitors. Kinetic characterization of these substitutions demonstrated an 80-fold reduction in the inhibition constant ( $K_i$ ) for the I50V single-mutant protease and a 270-fold-reduced  $K_i$  for the triple mutant M46I/I47V/I50V, compared to the wild-type enzyme

(Pazhanisamy S, St6uvr CM, Cullinan AB, Margolin N, Rao BG (1996) J. Biol. Chem. 271:17979-17985). The single mutants L10F, M46I, and I47V did not display reduced affinity for amprenavir. The catalytic efficiency ( $k_{cat}/K_m$ ) of the I50V mutant was decreased up to 25-fold, while the triple mutant M46I/I47V/I50V had a 2-fold-higher processing efficiency than the I50V single mutant, confirming the compensatory role of the M46I-and-I47V mutation. The reduced catalytic efficiency ( $k_{cat}/K_m$ ) for these mutants in processing peptides appeared to be due to both increased  $K_m$  and decreased  $k_{cat}$  values.

#### **VIRAL FITNESS**

The relative ability of a given virus or virus mutant to replicate is termed viral fitness. Fitness is dependent on both viral and host factors, including the genetic composition of the virus, the host immune response, and selective pressures such as the presence of anti-viral compounds. Many drug-resistant variants of HIV-1 are less fit than the wild-type, i.e. they grow more slowly in the absence of drug selection. However, since the replication of the wild-type virus is inhibited in the presence of drug, the resistant mutant can outgrow it. The reduction in fitness may be a result of several factors including: decreased ability of the mutated enzyme (i.e. PR or RT) to recognize its natural substrates, decreased stability of the mutant protein, or decreased kinetics of enzymatic catalysis. See Back et al., EMBO J. 15: 4040-4049, 1996; Goudsmit et al., J. Virol. 70: 5662-5664, 1996; Maschera et al., J. Biol. Chem. 271: 33231-33235, 1996; Croteau et al., J. Virol. 71: 1089-1096, 1997; Zennou et al., J. Virol. 72: 300-3306, 1998; Harrigan et al., J. Virol. 72:

3773-3778, 1998; Kosalaraksa et al., J. Virol. 73:  
5356-5363, 1999; Gerondelis et al., J. Virol. 73:  
5803-5813, 1999. Drug resistant viruses that are less fit  
than wild type may be less virulent i.e. they may cause  
5 damage to the host immune system more slowly than a wild  
type virus. Immunological decline may be delayed after the  
emergence of drug resistant mutants, compared to the rate  
of immunological decline in an untreated patient. The  
defect causing reductions in fitness may be partially or  
10 completely compensated for by the selection of viruses  
with additional amino acid substitutions in the same  
protein that bears the drug resistance mutations (for  
example, see Martinez-Picado et al., J. Virol.  
73:3744-3752, 1999), or in other proteins which interact  
15 with the mutated enzyme. Thus, amino acids surrounding the  
protease cleavage site in the gag protein may be altered  
so that the site is better recognized by a drug-resistant  
protease enzyme (Doyon et al., J. Virol. 70: 3763-3769,  
1996; Zhang et al., J. Virol. 71: 6662-6670, 1997; Mammano  
20 et al., J. Virol. 72: 7632-7637, 1998).

### INTEGRASE

Integration of viral DNA into the host chromosome is a  
necessary process in the HIV replication cycle (Brown,  
25 P.O., 1997, in *Retroviruses*; Coffin, J.M., Hughes, S.H. &  
Varmus, H.E., eds., Cold Spring Harbor Lab. Press,  
Plainview, NY, 161-203). The key steps of DNA integration  
are carried out by the viral integrase protein, which,  
along with protease and reverse transcriptase, is one of  
30 three enzymes encoded by HIV. Combination antiviral  
therapy with protease and reverse transcriptase inhibitors  
has demonstrated the potential therapeutic efficacy of



antiviral therapy for treatment for AIDS (Vandamme, A.M.,  
Van Vaerenbergh, K. & De Clerq, E., 1998, *Antiviral Chem.  
Chemother.* 9, 187-203). However, the ability of HIV to  
rapidly evolve drug resistance, together with toxicity  
5 problems, requires the development of additional classes  
of antiviral drugs. Integrase is an attractive target for  
antivirals because it is essential for HIV replication  
and, unlike protease and reverse transcriptase, there are  
no known counterparts in the host cell. Furthermore,  
10 integrase uses a single active site to accommodate two  
different configurations of DNA substrates, which may  
constrain the ability of HIV to develop drug resistance to  
integrase inhibitors. However, unlike protease and  
reverse transcriptase, for which several classes of  
15 inhibitors have been developed and cocrystal structures  
have been determined, progress with the development of  
integrase inhibitors has been slow. A major obstacle has  
been the absence of good lead compounds that can serve as  
the starting point for structure-based inhibitor  
20 development. Although numerous compounds have been  
reported to inhibit integrase activity *in vitro*, most of  
these compounds exhibit little specificity for integrase  
and are not useful as lead compounds (Pommier, Y., Pilon,  
A.A., Bajaj K, K., Mazumder, A. & Neamati, N., 1997,  
25 *Antiviral Chem. Chemother* 8).

HIV-1 integrase is a 32-kDa enzyme that carries out DNA  
integration in a two-step reaction (Brown, P.O., *ibid.*).  
In the first step, called 3' processing, two nucleotides  
30 are removed from each 3' end of the viral DNA made by  
reverse transcription. In the next step, called DNA  
strand transfer, a pair of transesterification reactions

integrates the ends of the viral DNA into the host genome. Integrase is comprised of three structurally and functionally distinct domains, and all three domains are required for each step of the integration reaction (Engelman, A. Bushman, F.D. & Craigie, R., 1993, *EMBO J.* 12, 3269-3275). The isolated domains form homodimers in solution, and the three-dimensional structures of all three separate dimers have been determined (Dyda, F., Hickman, A.B. Jenkins, T.M., Engelman, A., Craigie, R. & Davies, D.R., 1994, *Science* 226, 1981-1986; Goldgur, Y. Dyda, Hickman, A.B., Jenkins, T.M., Craigie, R. & Davies, D.R., 1998, *Proc. Natl. Acad. Sci., USA* 95, 9150-9154; Maignan, S., Guilloteau, J.P., Zhou-Liu, Q., Clement-Mella, C. & Mikol, V., 1998, *J Mol. Biol.* 282, 259-368; Lodi, P.J., Ernst, J.A., Kuszewski, J., Hickman, A.B., Engelman, A., Craigie, R., Clore, G.M. & Gronenborn, A.M., 1995 *Biochemistry* 34, 9826-9833; Eijkelenboom, A.P., Lutzke, R.A., Boelens, R., Plasterk, R.H., Kaptein, R. & Hard, K. 1995 *Nat. Struct. Biol.* 2, 807-810; Cai, M.L., Zheng, R., Caffrey, M., Craigie, R., Clore, G.M. & Gronenborn, A.M., 1997 *Nat. Struct. Biol.* 4, 839-840). Although little is known concerning the organization of these domains in the active complex with DNA substrates, integrase is likely to function as at least a tetramer (Dyda, F., Hickman, A.B. Jenkins, T.M., Engelman, A., Craigie, R. & Davies, D.R., 1994, *Science* 226, 1981-1986). Extensive mutagenesis studies mapped the catalytic site to the core domain (residues 50-212), which contains the catalytic residues D64, D116, and E152 (Engelman, A. & Craigie R., 1992, *J. Virol.* 66, 6361-6369; Kulkosky, J., Jones, K.S., Katz, R.A., Mack, J.P. & Skalka, A.M., 1992, *Mol. Cell Biol* 12, 2331-2338). The structure of this

domain of HIV-1 integrase has been determined in several crystal forms (Dyda, F., Hickman, A.B. Jenkins, T.M., Engelman, A., Craigie, R. & Davies, D.R., 1994, *Science* 226, 1981-1986; Goldgur, Y. Dyda, Hickman, A.B., Jenkins, T.M., Craigie, R. & Davies, D.R., 1998, *Proc. Natl. Acad. Sci., USA* 95, 9150-9154; Maignan, S., Guilloteau, J.P., Zhou-Liu, Q., Clement-Mella, C. & Mikol, V., 1998, *J Mol. Biol.* 282, 259-368).

10 Hazuda et al. (*Science* 287: 646-650, 2000) have described compounds (termed L-731, 988 and L-708,906) which specifically inhibit the strand-transfer activity of HIV-1 integrase and HIV-1 replication in vitro. Viruses grown in the presence of these inhibitors display reduced inhibitor susceptibility and bear mutations in the integrase coding region at amino acid positions 66 (T66I), 153 (S153Y), and 154 (M154I). Site-directed mutants of a laboratory strain of HIV-1 (HXB2) with these amino acid changes confirmed their direct role in conferring reduced integrase inhibitor susceptibility. In addition some of these mutants displayed delayed growth kinetics, suggesting that viral fitness was impaired.

25 It is an object of this invention to provide a drug susceptibility and resistance test capable of showing whether a viral population in a patient is either more or less susceptible to a given prescribed drug. Another object of this invention is to provide a test that will enable the physician to substitute one or more drugs in a therapeutic regimen for viruses that show altered susceptibility to a given drug or drugs after a course of therapy. Yet another object of this invention is to

provide a test that will enable selection of an effective drug regimen for the treatment of HIV infections and/or AIDS. Yet another object of this invention is to provide the means for identifying alterations in the drug susceptibility profile of a patient's virus, in particular identifying changes in susceptibility to protease inhibitors. Still another object of this invention is to provide a test and methods for evaluating the biological effectiveness of candidate drug compounds which act on specific viruses, viral genes and/or viral proteins particularly with respect to alterations in viral drug susceptibility associated with protease inhibitors. It is also an object of this invention to provide the means and compositions for evaluating HIV antiretroviral drug resistance and susceptibility.

It is an object of this invention to provide a method for measuring replication fitness which can be adapted to viruses, including, but not limited to human immunodeficiency virus (HIV), hepadnaviruses (human hepatitis B virus), flaviviruses (human hepatitis C virus) and herpesviruses (human cytomegalovirus). This and other objects of this invention will be apparent from the specification as a whole.

Summary of the Invention

The present invention relates to methods of monitoring, via phenotypic and genotypic methods the clinical progression of human immunodeficiency virus infection and its response to antiviral therapy. The invention is also based, in part, on the discovery that genetic changes in HIV protease (PR) which confer changes in susceptibility to antiretroviral therapy may be rapidly determined directly from patient plasma HIV RNA using phenotypic or genotypic methods. The methods utilize nucleic acid amplification based assays, such as polymerase chain reaction (PCR). Herein—after, such nucleic acid amplification based assays will be referred to as PCR based assays. This invention is based in part on the discovery of mutations at codons 10, 20, 36, 46, 63, 77 and 88 of HIV protease in PRI treated patients in which the presence of certain combinations of these mutations correlate with changes in certain PRI susceptibilities. This invention is also based on the discovery that susceptibility to HIV protease antivirals may not be altered even if primary mutations are present. Additional mutations at secondary positions in HIV protease are required for a reduction in virus susceptibility. This invention established for the first time that a mutation at position 82 of protease (V82A, F, S, or T) in the absence of another primary mutation was not correlated with a reduction in drug susceptibility. Decreased susceptibility to protease inhibitors, such as indinavir and saquinavir, in viruses containing V82A, F, S or T was observed in viruses with additional mutations at secondary positions, such as, 24, 71, 54, 46, 10 and/or 63 as described herein. Decreased susceptibility to protease

inhibitors, such as indinavir and saquinavir, in viruses containing V82A, F, S or T was also observed in viruses with at least 3 or more additional mutations at secondary positions. This inventions also established for the first time that a mutation at position 90 of protease (L90M) in the absence of another primary mutation was not correlated with a reduction in drug susceptibility. Decreased susceptibility to protease inhibitors, such as indinavir and saquinavir, in viruses containing L90M was observed in viruses with additional mutations at secondary positions, such as, 73, 71, 77, and/or 10 as described herein. Decreased susceptibility to protease inhibitors, such as indinavir and saquinavir, in viruses containing L90M was also observed in viruses with at least 3 or more additional mutations at secondary positions. The mutations were found in plasma HIV nucleic acid after a period of time following the initiation of therapy. The development of these mutations, or combinations of these mutations, in HIV PR was found to be an indicator of the development of alterations in phenotypic susceptibility/resistance, which can be associated with virologic failure and subsequent immunological response.

In one embodiment of the invention, a method of assessing the effectiveness of protease antiretroviral therapy of an HIV-infected patient is provided comprising: (a) collecting a plasma sample from the HIV-infected patient; (b) evaluating whether the plasma sample contains nucleic acid encoding HIV protease having a mutation at primary and secondary positions; and (c) determining changes in susceptibility to a protease inhibitor.

In a further embodiment of the invention, PCR based assays, including phenotypic and genotypic assays, may be used to detect a substitution at codon 88 from asparagine to a serine residue either alone or in combination with one or more mutations at other codons selected from the group consisting of 10, 20, 36, 46, 63 and/or 77 or a combination thereof of HIV PR. A mutation at codon 88 from an asparagine residue to a serine residue (N88S) alone correlates with an increase in susceptibility to amprenavir and a mutation at codon 88 from an asparagine residue to a serine residue in combination with mutations at codons 63 and/or 77 or a combination thereof correlates with an increase in susceptibility to amprenavir and a decrease in nelfinavir and indinavir susceptibility.

In a further embodiment of the invention, PCR based assays, including phenotypic and genotypic assays, may be used to detect mutations at codons 10, 20, 36, 46, 63, 77, and 88 of HIV PR which correlate with changes in susceptibility to antiretroviral therapy and immunologic response. Once mutations at these loci have been detected in a patient undergoing PRI antiretroviral therapy, an alteration in the therapeutic regimen should be considered. The timing at which a modification of the therapeutic regimen should be made, following the assessment of antiretroviral therapy using PCR based assays, may depend on several factors including the patient's viral load, CD4 count, and prior treatment history.

In a further embodiment of the invention, PCR based assays, including phenotypic and genotypic assays, may be



used to detect a substitution at codon 82 from valine to an alanine (V82A), phenylalanine (V82F), serine (V82S), or threonine (V82T) residue either alone or in combination with one or more mutations at other codons, referred to herein as secondary mutations, selected from the group consisting of 20, 24, 36, 71, 54, 46, 63 and/or 10 or a combination thereof of HIV PR. A mutation at codon 82 from a valine residue to a alanine, phenylalanine, serine or threonine alone correlates with susceptibility to certain protease inhibitors including indinavir and saquinavir. A mutation at codon 82 from a valine residue to a alanine, phenylalanine, serine or threonine in combination with secondary mutations at codons 24 and/or 71 or 20 and/or 36 correlates with a reduction in susceptibility to indinavir and saquinavir, respectively. A mutation at codon 82 from a valine residue to a alanine, phenylalanine, serine or threonine in combination with at least 3 secondary mutations correlates with a reduction in susceptibility to indinavir and saquinavir.

In a further embodiment of the invention, PCR based assays, including phenotypic and genotypic assays, may be used to detect a substitution at codon 90 from leucine to a methionine (L90M) residue either alone or in combination with one or more mutations at other codons, referred to herein as secondary mutations, selected from the group consisting of 73, 71, 46 and/or 10 or a combination thereof of HIV PR. A mutation at codon 90 from a leucine residue to a methionine alone correlates with susceptibility to certain protease inhibitors including indinavir and saquinavir. A mutation at codon 90 from a leucine residue to a methionine in combination with



secondary mutations at codons 73 and/or 71 or 73, 71 and/or 77 correlates with a reduction in susceptibility to indinavir and saquinavir, respectively. A mutation at codon 90 from a leucine residue to a methionine in combination with at least 3 secondary mutations correlates with a reduction in susceptibility to indinavir and saquinavir.

In another aspect of the invention there is provided a method for assessing the effectiveness of a protease inhibitor antiretroviral drug comprising: (a) introducing a resistance test vector comprising a patient-derived segment and an indicator gene into a host cell; (b) culturing the host cell from step (a); (c) measuring expression of the indicator gene in a target host cell wherein expression of the indicator gene is dependent upon the patient derived segment; and (d) comparing the expression of the indicator gene from step (c) with the expression of the indicator gene measured when steps (a) - (c) are carried out in the absence of the PRI anti-HIV drug, wherein a test concentration of the PRI, anti-HIV drug is presented at steps (a) - (c); at steps (b) - (c); or at step (c).

This invention also provides a method for assessing the effectiveness of protease inhibitor antiretroviral therapy in a patient comprising: (a) developing a standard curve of drug susceptibility for an PRI anti-HIV drug; (b) determining PRI anti-HIV drug susceptibility in the patient using the susceptibility test described above; and (c) comparing the PRI anti-HIV drug susceptibility in step (b) with the standard curve determined in step (a), wherein a decrease in PRI anti-HIV susceptibility

indicates development of anti-HIV drug resistance in the patient's virus and an increase in PRI anti-HIV susceptibility indicates drug hypersensitivity in the patient's virus.

5

10 This invention also provides a method for evaluating the biological effectiveness of a candidate PRI HIV antiretroviral drug compound comprising: (a) introducing a resistance test vector comprising a patient-derived segment and an indicator gene into a host cell; (b) culturing the host cell from step (a); (c) measuring expression of the indicator gene in a target host cell wherein expression of the indicator gene is dependent upon the patient derived segment; and (d) comparing the  
15 expression of the indicator gene from step (c) with the expression of the indicator gene measured when steps (a) - (c) are carried out in the absence of the candidate PRI anti-viral drug compound, wherein a test concentration of the candidate PRI anti-viral drug compound is present at  
20 steps (a) - (c); at steps (b) - (c); or at step (c).

The expression of the indicator gene in the resistance test vector in the target cell is ultimately dependent upon the action of the HIV enzymes (PR and RT) encoded by  
25 the patient-derived segment DNA sequences. The indicator gene may be functional or non-functional.

In another aspect this invention is directed to antiretroviral drug susceptibility and resistance tests  
30 for HIV/AIDS. Particular resistance test vectors of the invention for use in the HIV/AIDS antiretroviral drug susceptibility and resistance test are identified.

Yet another aspect of this invention provides for the identification and assessment of the biological effectiveness of potential therapeutic antiretroviral compounds for the treatment of HIV and/or AIDS. In another aspect, the invention is directed to a novel resistance test vector comprising a patient-derived segment further comprising one or more mutations on the PR gene and an indicator gene.

10

Still another aspect of this invention provides for the identification and assessment of the fitness of a virus infecting a patient. In another aspect, the invention is directed to a novel resistance test vector comprising a patient-derived segment further comprising one or more mutations on the PR gene and an indicator gene, enabling the measurement of viral fitness.

15

For the purpose of this document, the word "and/or" shall mean "at least one of the following."

Brief Description of the Drawings

Fig. 1

Resistance Test Vector. A diagrammatic representation of  
5 the resistance test vector comprising a patient derived  
segment and an indicator gene.

Fig. 2

Two Cell Assay. Schematic Representation of the Assay. A  
10 resistance test vector is generated by cloning the  
patient-derived segment into an indicator gene viral  
vector. The resistance test vector is then co-transfected  
with an expression vector that produces amphotropic murine  
leukemia virus (MLV) envelope protein or other viral or  
15 cellular proteins which enable infection. Pseudotyped  
viral particles are produced containing the protease (PR)  
and the reverse transcriptase (RT) gene products encoded  
by the patient-derived DNA sequences. The particles are  
then harvested and used to infect fresh cells. Using  
20 defective PR and RT sequences it was shown that luciferase  
activity is dependent on functional PR and RT. PR  
inhibitors are added to the cells following transfection  
and are thus present during particle maturation. RT  
inhibitors, on the other hand, are added to the cells at  
25 the time of or prior to viral particle infection. The  
assay is performed in the absence of drug and in the  
presence of drug over a wide range of concentrations.  
Luciferase activity is determined and the percentage (%)  
inhibition is calculated at the different drug  
30 concentrations tested.

Fig. 3

Examples of phenotypic drug susceptibility profiles. Data are analyzed by plotting the percent inhibition of luciferase activity vs. log10 concentration. This plot is used to calculate the drug concentration that is required to inhibit virus replication by 50% (IC50) or by 95% (IC95). Shifts in the inhibition curves towards higher drug concentrations are interpreted as evidence of drug resistance. Three typical curves for a nucleoside reverse transcriptase inhibitor (AZT), a non-nucleoside reverse transcriptase inhibitor (efavirenz), and a protease inhibitor (indinavir) are shown. A reduction in drug susceptibility (resistance) is reflected in a shift in the drug susceptibility curve toward higher drug concentrations (to the right) as compared to a baseline (pre-treatment) sample or a drug susceptible virus reference control, such as pNL4-3 or HXB-2, when a baseline sample is not available.

Fig. 4

Phenotypic PRI susceptibility profile: patient 0732. A PCR-based phenotypic susceptibility assay was carried out giving the phenotypic drug susceptibility profile showing decreased susceptibility to nelfinavir and indinavir, and increased susceptibility to amprenavir.

Fig. 5

Phenotypic PRI susceptibility profile of a protease mutant generated by site-specific oligonucleotide-directed mutagenesis. A PCR-based phenotypic susceptibility assay was carried out giving the phenotypic drug susceptibility profile of a virus having substitutions at codons 63, 77

and 88 (L63P, V77I and N88S). The profile demonstrates resistance to both nelfinavir and indinavir, and increased susceptibility to amprenavir.

- 5 Figure 6. Distribution of saquinavir hyper-susceptibility by amino acid change at position 82.

Figure 7. Relative luciferase activity of integrase inhibitor-resistant site-directed mutants.

10

Fig. A

- Two Cell Fitness Assay. Schematic Representation of the Fitness Assay. A fitness test vector is generated by cloning the patient-derived segment into an indicator gene viral vector. The fitness test vector is then co-transfected with an expression vector that produces amphotropic murine leukemia virus (MLV) envelope protein or other viral or cellular proteins which enable infection. Pseudotyped viral particles are produced containing the protease (PR) and the reverse transcriptase (RT) gene products encoded by the patient-derived DNA sequences. The particles are then harvested and used to infect fresh cells. Using defective PR and RT sequences it was shown that luciferase activity is dependent on functional PR and RT. The fitness assay is typically performed in the absence of drug. If desired, the assay can also be performed at defined drug concentrations. Luciferase activity produced by patient derived viruses is compared to the luciferase activity produced by well-characterized reference viruses. Replication fitness is expressed as a percent of the reference.
- 15
- 20
- 25
- 30

Figure B.

Determining the replication fitness of patient viruses. Virus stocks produced from fitness test vectors derived from patient samples were used to infect cells. Luciferase activity was measured at various times after infection. Patient derived viruses may produce more, approximately the same, or less luciferase activity than the reference virus (Ref) and are said to have greater, equivalent, or reduced replication fitness, respectively. The drug susceptibility profiles of three representative patient derived viruses are shown (P1, P2, P3).

Figure C.

Identifying alterations in protease or reverse transcriptase function associated with differences in replication fitness of patient viruses. Replication fitness is expressed as a percent of the reference virus (top). Fitness measurements are compared to protease processing of the p55 gag polyprotein (middle) and reverse transcriptase activity (bottom). Protease processing is measured by Western blot analysis using an antibody that reacts with the mature capsid protein (p24). The detection of unprocessed p55 or incompletely processed p41 polyproteins are indicators of reduced cleavage. Reverse transcriptase activity is measured using a quantitative RT-PCR assay and is expressed as a percent of the reference virus.

Figure D.

Correlating reduced replication fitness with reduced reverse transcriptase activity. Viruses containing

various amino acid substitutions at position 190 (A, S, C, Q, E, T, V) of reverse transcriptase were constructed using site directed mutagenesis. The reference virus contains G at this position. Replication fitness and

5

reverse transcriptase activities were compared.

Figure E.

Correlating reduced replication fitness with reduced  
10 protease processing of p55 gag. Viruses containing various amino acid substitutions in protease (D30N, L90M, etc) were constructed using site directed mutagenesis. Replication fitness and p55 gag processing were compared.

15 Figure F.

Correlating reduced replication fitness with reduced drug  
susceptibility. A large collection (n=134) of patient  
samples were evaluated for phenotypic drug susceptibility  
and replication fitness. Replication fitness and drug  
20 susceptibility were compared.

Figure G.

Relationship between protease inhibitor susceptibility and  
replication fitness. Patient samples were sorted based on  
25 their replication fitness (<25% of reference, 26-75% of reference, and >75% of reference). Mean values for protease inhibitor susceptibility were determined for each fitness group and plotted for each drug and all drugs combined.

30

Figure H.

Relationship between reverse transcriptase inhibitor



susceptibility and replication fitness. Patient samples were sorted based on their replication fitness (<25% of reference, 26-75% of reference, and >75% of reference). Mean values for reverse transcriptase susceptibility were determined for each fitness group and plotted for each drug and all drugs combined.

Figure I.

Reduced replication fitness is associated with high numbers of protease mutations, and the L90M mutation. Patient viruses were sorted based on the number of protease mutations. Viruses with large numbers of protease mutations or the L90M protease mutation generally exhibit reduced replication fitness.

Figure J.

Low replication capacity is associated with specific protease mutations. Patient viruses were sorted based on replication capacity. Specific protease mutations either alone (D30N) or in combination (L90M plus others) were observed with high frequency in viruses with reduced replication fitness.

Figure K.

Relationship between nelfinavir susceptibility, protease processing and replication fitness. Patient viruses were sorted based on nelfinavir susceptibility (<10 or >10 of reference). Protease processing and replication fitness were plotted for all patient viruses. Viruses with reduced nelfinavir susceptibility generally exhibited reduced protease processing and reduced replication fitness.

Figure L. Protease mutations associated with reduced protease processing. Patient viruses were sorted based on protease processing. Specific protease mutations were observed at high frequency in viruses with reduced protease processing.

Figure M.  
Representative patient sample exhibiting reversion to drug susceptibility during a period of drug treatment interruption. Virus samples were collected weekly during a period of treatment interruption and evaluated for phenotypic drug susceptibility. Values shown represent fold change in susceptibility compared to the reference virus.

Figure N.  
Representative patient sample exhibiting increased replication fitness during a period of drug treatment interruption. Virus samples were collected weekly during a period of treatment interruption and evaluated for phenotypic drug susceptibility. Fitness values shown represent percent of the reference virus. The increase in fitness between week 9 and week 10 corresponds to improved protease processing (bottom) and reversion of the drug resistant phenotype to a drug sensitive phenotype (Figure M).

Figure O.  
Increased replication fitness during treatment interruption. Replication fitness was measured at the time of treatment interruption and various times during

the period of treatment interruption. Generally, replication fitness was significantly higher in samples that corresponded to timepoints after the virus had reverted from a drug resistant phenotype to a drug sensitive phenotype.

#### Detailed Description of the Invention

10 The present invention relates to methods of monitoring the clinical progression of HIV infection in patients receiving antiretroviral therapy, particularly protease inhibitor antiretroviral therapy.

15 In one embodiment, the present invention provides for a method of evaluating the effectiveness of antiretroviral therapy of a patient comprising (i) collecting a biological sample from an HIV-infected patient; and (ii) determining whether the biological sample comprises  
20 nucleic acid encoding HIV PR having a mutation at one or more positions in the PR. The mutation(s) correlate positively with alterations in phenotypic susceptibility.

In a specific embodiment, the invention provides for a  
25 method of evaluating the effectiveness of PRI antiretroviral therapy of a patient comprising (i) collecting a biological sample from an HIV-infected patient; and (ii) determining whether the biological sample comprises nucleic acid encoding HIV PR having a  
30 mutation at codon 88 from an asparagine residue to a serine residue (N88S). This invention established, using a phenotypic susceptibility assay, that a mutation at

codon 88 to a serine residue of HIV protease is correlated with an increase in amprenavir susceptibility.

In a specific embodiment, the invention provides for a method of evaluating the effectiveness of PRI antiretroviral therapy of a patient comprising (i) collecting a biological sample from an HIV-infected patient; and (ii) determining whether the biological sample comprises nucleic acid encoding HIV PR having a mutation at codon 88 from an asparagine residue to a serine residue (N88S) either alone or in combination with mutations at codons 63 and/or 77 or a combination thereof. This invention established, using a phenotypic susceptibility assay, that a mutation at codon 88 to a serine residue of HIV protease is correlated with an increase in amprenavir susceptibility and a mutation at codon 88 to a serine residue in combination with mutations at codons 63 and/or 77 or a combination thereof of HIV protease are correlated with an increase in amprenavir susceptibility and a decrease in nelfinavir and indinavir susceptibility.

In a specific embodiment, the invention provides for a method of evaluating the effectiveness of PRI antiretroviral therapy of a patient comprising (i) collecting a biological sample from an HIV-infected patient; and (ii) determining whether the biological sample comprises nucleic acid encoding HIV PR having a mutation at codon 88 from an asparagine residue to a serine residue (N88S) either alone or in combination with mutations at codons 46, 63 and/or 77 or a combination thereof. This invention established, using a phenotypic

susceptibility assay, that a mutation at codon 88 to a serine residue of HIV protease is correlated with an increase in amprenavir susceptibility and a mutation at codon 88 to a serine residue in combination with mutations at codons 46, 63 and/or 77 or a combination thereof of HIV protease are correlated with an increase in amprenavir susceptibility and a decrease in nelfinavir and indinavir susceptibility.

10 In a specific embodiment, the invention provides for a method of evaluating the effectiveness of PRI antiretroviral therapy of a patient comprising (i) collecting a biological sample from an HIV-infected patient; and (ii) determining whether the biological sample comprises nucleic acid encoding HIV PR having a mutation at codon 88 from an asparagine residue to a serine residue (N88S) either alone or in combination with mutations at codons 10, 20, 36, 46, 63 and/or 77 or a combination thereof. This invention established, using a phenotypic susceptibility assay, that a mutation at codon 88 to a serine residue of HIV protease is correlated with an increase in amprenavir susceptibility and a mutation at codon 88 to a serine residue in combination with mutations at codons 10, 20, 36, 46, 63 and/or 77 or a combination thereof of HIV protease are correlated with an increase in amprenavir susceptibility and a decrease in nelfinavir and indinavir susceptibility.

Under the foregoing circumstances, the phenotypic susceptibility profile and genotypic profile of the HIV virus infecting the patient has been altered reflecting a change in response to the antiretroviral agent. In the

case of PRI antiretroviral therapy, the HIV virus infecting the patient may be resistant to one or more PRIs but hypersensitive to another of the PRIs as described herein. It therefore may be desirable after detecting the mutation(s), to either increase the dosage of the antiretroviral agent, change to another antiretroviral agent, or add one or more additional antiretroviral agents to the patient's therapeutic regimen. For example, if the patient was being treated with nelfinavir when the N88S mutation arose, the patient's therapeutic regimen may desirably be altered by either (i) changing to a different PRI antiretroviral agent, such as saquinavir, ritonavir or amprenavir and stopping nelfinavir treatment; or (ii) increasing the dosage of nelfinavir; or (iii) adding another antiretroviral agent to the patient's therapeutic regimen. The effectiveness of the modification in therapy may be further evaluated by monitoring viral burden such as by HIV RNA copy number. A decrease in HIV RNA copy number correlates positively with the effectiveness of a treatment regimen.

The phrase "correlates positively," as used herein, indicates that a particular result renders a particular conclusion more likely than other conclusions.

When reference is made to a particular codon number, it is understood that the codon number refers to the position of the amino acid that the codon codes for. Therefore a codon referencing a particular number is equivalent to a "postion" referencing a particular number, such as for example, "codon 88" or "position 88".

Another preferred, non-limiting, specific embodiment of the invention is as follows: A method of evaluating the effectiveness of PRI therapy of a patient comprising (i) collecting a biological sample from an HIV-infected patient; (ii) purifying and converting the viral RNA to cDNA and amplifying HIV sequences using HIV primers that result in a PCR product that comprises the PR gene; (iii) performing PCR using primers that result in PCR products comprising wild type or serine at codon 88; and (iv) determining, via the products of PCR, the presence or absence of a serine residue at codon 88.

Another preferred, non-limiting, specific embodiment of the invention is as follows: A method of evaluating the effectiveness of PRI therapy of a patient comprising (i) collecting a biological sample from an HIV-infected patient; (ii) purifying and converting the viral RNA to cDNA and amplifying HIV sequences using HIV primers that result in a PCR product that comprises the PR gene; (iii) performing PCR using primers that result in PCR products comprising wild type or serine at codon 88 and mutations at codons 63 and/or 77; and (iv) determining, via the products of PCR, the presence or absence of a serine residue at codon 88 and the presence or absence of mutations at codons 63 and/or 77.

Another preferred, non-limiting, specific embodiment of the invention is as follows: A method of evaluating the effectiveness of PRI therapy of a patient comprising (i) collecting a biological sample from an HIV-infected patient; (ii) purifying and converting the viral RNA to cDNA and amplifying HIV sequences using HIV primers that

result in a PCR product that comprises the PR gene; (iii) performing PCR using primers that result in PCR products comprising wild type or serine at codon 88 and mutations at codons 63, 77 and/or 46 or a combination thereof; and  
5 (iv) determining, via the products of PCR, the presence or absence of a serine residue at codon 88 and the presence or absence of mutations at codons 63, 77 and/or 46 or a combination thereof.

10 Another preferred, non-limiting, specific embodiment of the invention is as follows: A method of evaluating the effectiveness of PRI therapy of a patient comprising (i) collecting a biological sample from an HIV-infected patient; (ii) purifying and converting the viral RNA to  
15 cDNA and amplifying HIV sequences using HIV primers that result in a PCR product that comprises the PR gene; (iii) performing PCR using primers that result in PCR products comprising wild type or serine at codon 88 and mutations at codons 63, 77, 46, 10, 20, and/or 36 or a combination  
20 thereof; and (iv) determining, via the products of PCR, the presence or absence of a serine residue at codon 88 and the presence or absence of mutations at codons 63, 77, 46, 10, 20, and/or 36 or a combination thereof.

25 The presence of the mutation at codon 88 to a serine of HIV PR indicates that the effectiveness of the current or prospective PRI therapy may require alteration, since as shown by this invention mutation at codon 88 to a serine residue increases the susceptibility to amprenavir. Using  
30 the methods of this invention, changes in the PRI therapy would be indicated.



The presence of the mutation at codon 88 to a serine of alone or in combination with mutations at condons 63, 77, 46, 10, 20, and/or 36 or a combination thereof of HIV PR indicates that the effectiveness of the current or prospective PRI therapy may require alteration, since as shown by this invention a mutation at codon 88 to a serine residue alone increases the susceptibility to amprenavir and a mutation at codon 88 to a serine residue in combination with mutations at condons 63, 77, 46, 10, 20, and/or 36 or a combination increases the susceptibility to amprenavir but also reduces the susceptibility to nelfinavir and indinavir. Using the methods of this invention, changes in the PRI therapy would be indicated.

Another preferred, non-limiting, specific embodiment of the invention is as follows: a method of evaluating the effectiveness of antiretroviral therapy of an HIV-infected patient comprising: (a) collecting a biological sample from an HIV-infected patient; and (b) determining whether the biological sample comprises nucleic acid encoding HIV protease having a mutation at codon 88 to serine. Using the phenotypic susceptibility assay, it was observed that the presence of the mutation at codon 88 to serine of HIV PR causes a an increase in amprenavir susceptibility.

Another preferred, non-limiting, specific embodiment of the invention is as follows: a method of evaluating the effectiveness of antiretroviral therapy of an HIV-infected patient comprising: (a) collecting a biological sample from an HIV-infected patient; and (b) determining whether the biological sample comprises nucleic acid encoding HIV protease having a mutation at codon 88 to serine and

additional mutation(s) at codons 63 and/or 77 or a combination thereof. Using the phenotypic susceptibility assay, it was observed that the presence of the mutation at codon 88 to serine of HIV PR causes an increase in amprenavir susceptibility and the presence of the mutations at codon 88 to serine in combination with a mutation at codon(s) 63 and/or 77 or a combination thereof of HIV PR causes a decrease in nelfinavir and indinavir susceptibility while increasing amprenavir susceptibility.

Another preferred, non-limiting, specific embodiment of the invention is as follows: a method of evaluating the effectiveness of antiretroviral therapy of an HIV-infected patient comprising: (a) collecting a biological sample from an HIV-infected patient; and (b) determining whether the biological sample comprises nucleic acid encoding HIV protease having a mutation at codon 88 to serine and additional mutation(s) at codons 63, 77 and/or 46 or a combination thereof. Using the phenotypic susceptibility assay, it was observed that the presence of the mutation at codon 88 to serine of HIV PR causes an increase in amprenavir susceptibility and the presence of the mutations at codon 88 to serine in combination with a mutation at codon(s) 46, 63 and/or 77 or a combination thereof of HIV PR causes a decrease in nelfinavir and indinavir susceptibility while increasing amprenavir susceptibility.

Another preferred, non-limiting, specific embodiment of the invention is as follows: a method of evaluating the effectiveness of antiretroviral therapy of an HIV-infected patient comprising: (a) collecting a biological sample

from an HIV-infected patient; and (b) determining whether the biological sample comprises nucleic acid encoding HIV protease having a mutation at codon 88 to serine and additional mutation(s) at codons 63, 77, 46, 10, 20 and/or 36 or a combination thereof. Using the phenotypic susceptibility assay, it was observed that the presence of the mutation at codon 88 to serine of HIV PR causes an increase in amprenavir susceptibility and the presence of the mutations at codon 88 to serine in combination with a mutation at codon(s) 63, 77, 46, 10, 20 and/or 36 or a combination thereof of HIV PR causes a decrease in nelfinavir and indinavir susceptibility while increasing amprenavir susceptibility.

This invention also provides the means and methods to use the resistance test vector comprising an HIV gene and further comprising a PR mutation for drug screening. More particularly, the invention describes the resistance test vector comprising the HIV protease having a mutation at codon 88 to a serine alone or in combination with mutations at codons 10, 20, 36, 46, 63 and/or 77 or a combination thereof for drug screening. The invention further relates to novel vectors, host cells and compositions for isolation and identification of the HIV-1 protease inhibitor resistant mutant and using such vectors, host cells and compositions to carry out anti-viral drug screening. This invention also relates to the screening of candidate drugs for their capacity to inhibit said mutant.

30

This invention provides a method for identifying a compound which is capable of affecting the function of the

protease of HIV-1 comprising contacting the compound with the polypeptide(s) comprising all or part of the HIV-1 protease, wherein codon 88 is changed to a serine residue, wherein a positive binding indicates that the compound is capable of affecting the function of said protease.

This invention also provides a method for assessing the viral fitness of patient's virus comprising: (a) determining the luciferase activity in the absence of drug for the reference control using the susceptibility test described above; (b) determining the luciferase activity in the absence of drug for the patient virus sample using the susceptibility test described above; and (c) comparing the luciferase activity determined in step (b) with the luciferase activity determined in step (a), wherein a decrease in luciferase activity indicates a reduction in viral fitness of the patient's virus.

If a resistance test vector is constructed using a patient derived segment from a patient virus which is unfit, and the fitness defect is due to genetic alterations in the patient derived segment, then the virus produced from cells transfected with the resistance test vector will produce luciferase more slowly. This defect will be manifested as reduced luciferase activity (in the absence of drug) compared to the drug sensitive reference control, and may be expressed as a percentage of the control.

In a further embodiment of the invention, PCR based assays, including phenotypic and genotypic assays, may be used to detect mutations at positions 20 and 88 of HIV PR, which correlate with a reduction in viral fitness and

immunological response.

It is a further embodiment of this invention to provide a means and method for measuring replication fitness for  
5 viruses, including, but not limited to human immunodeficiency virus (HIV), hepadnaviruses (human hepatitis B virus), flaviviruses (human hepatitis C virus) and herpesviruses (human cytomegalovirus).

10 This invention further relates to a means and method for measuring the replication fitness of HIV-1 that exhibits reduced drug susceptibility to reverse transcriptase inhibitors and protease inhibitors.

15 In a further embodiment of the invention , a means and methods are provided for measuring replication fitness for other classes of inhibitors of HIV-1 replication, including, but not limited to integration, virus assembly, and virus attachment and entry.

20 This invention relates to a means and method for identifying mutations in protease or reverse transcriptase that alter replication fitness.

25 In a further embodiment of the invention , a means and methods are provided for identifying mutations that alter replication fitness for other components of HIV-1 replication, including, but not limited to integration, virus assembly, and virus attachment and entry.

30

This invention also relates to a means and method for quantifying the affect that specific mutations in protease or reverse transcriptase have on replication fitness.

5 In a further embodiment of the invention , a means and method are provided for quantifying the affect that specific protease and reverse transcriptase mutations have on replication fitness in other viral genes involved in HIV-1 replication, including, but not limited to the gag,  
10 pol, and envelope genes.

This invention also relates to the high incidence of patient samples with reduced replication fitness.

15 This invention relates to the correlation between reduced drug susceptibility and reduced replication fitness.

This invention further relates to the occurrence of viruses with reduced fitness in patients receiving  
20 protease inhibitor and/or reverse transcriptase inhibitor treatment.

This invention further relates to the incidence of patient samples with reduced replication fitness in which the  
25 reduction in fitness is due to altered protease processing of the gag polyprotein (p55).

This invention further relates to the incidence of protease mutations in patient samples that exhibit low,  
30 moderate or normal (wildtype) replication fitness.

This invention further relates to protease mutations that are frequently observed, either alone or in combination, in viruses that exhibit reduced replication capacity.

- 5 This invention also relates to the incidence of patient samples with reduced replication fitness in which the reduction in fitness is due to altered reverse transcriptase activity. This invention relates to the occurrence of viruses with reduced replication fitness in
- 10 patients failing antiretroviral drug treatment. This invention further relates to a means and method for using replication fitness measurements to guide the treatment of HIV-1. This invention further relates to a means and method for using replication fitness measurements to guide
- 15 the treatment of patients failing antiretroviral drug treatment. This invention further relates to the means and methods for using replication fitness measurements to guide the treatment of patients newly infected with HIV-1.
- 20 This invention, provides the means and methods for using replication fitness measurements to guide the treatment of viral diseases, including, but not limited to HIV-1, hepadnaviruses (human hepatitis B virus), flaviviruses (human hepatitis C virus) and herpesviruses (human
- 25 cytomegalovirus).

In a further embodiment, the invention provides a method for determining replication capacity for a patient's virus comprising:

30

- (a) introducing a resistance test vector comprising a patient derived segment and an indicator gene into a host cell;

(b) culturing the host cell from (a);

(c) harvesting viral particles from step (b) and  
infecting target host cells;

5

(d) measuring expression of the indicator gene in  
the target host cell, wherein the expression of  
the indicator gene is dependent upon the  
patient-derived segment;

10

(e) comparing the expression of the indicator gene  
from (d) with the expression of the indicator  
gene measured when steps (a) through (d) are  
carried out in a control resistance test vector;  
and

15

(f) normalizing the expression of the indicator gene  
by measuring an amount of virus in step (c).

20

As used herein, "patient-derived segment" encompasses  
segments derived from human and various animal species.  
Such species include, but are not limited to chimpanzees,  
horses, cattles, cats and dogs.

25

Patient-derived segments can also be incorporated into  
resistance test vectors using any of several alternative  
cloning techniques as set forth in detail in US Patent  
Number 5,837,464 (International Publication Number WO  
97/27319) which is hereby incorporated by reference. For  
example, cloning via the introduction of class II  
restriction sites into both the plasmid backbone and the  
patient-derived segments or by uracil DNA glycosylase  
primer cloning.

30



The patient-derived segment may be obtained by any method of molecular cloning or gene amplification, or modifications thereof, by introducing patient sequence acceptor sites, as described below, at the ends of the patient-derived segment to be introduced into the resistance test vector. For example, in a gene amplification method such as PCR, restriction sites corresponding to the patient-sequence acceptor sites can be incorporated at the ends of the primers used in the PCR reaction. Similarly, in a molecular cloning method such as cDNA cloning, said restriction sites can be incorporated at the ends of the primers used for first or second strand cDNA synthesis, or in a method such as primer-repair of DNA, whether cloned or uncloned DNA, said restriction sites can be incorporated into the primers used for the repair reaction. The patient sequence acceptor sites and primers are designed to improve the representation of patient-derived segments. Sets of resistance test vectors having designed patient sequence acceptor sites provide representation of patient-derived segments that may be underrepresented in one resistance test vector alone.

"Resistance test vector" means one or more vectors which taken together contain DNA comprising a patient-derived segment and an indicator gene. Resistance test vectors are prepared as described in US Patent Number 5,837,464 (International Publication Number WO 97/27319), which is hereby incorporated by reference, by introducing patient sequence acceptor sites, amplifying or cloning patient-derived segments and inserting the amplified or cloned sequences precisely into indicator gene viral

vectors at the patient sequence acceptor sites. Alternatively, a resistance test vector (also referred to as a resistance test vector system) is prepared by introducing patient sequence acceptor sites into a packaging vector, amplifying or cloning patient-derived segments and inserting the amplified or cloned sequences precisely into the packaging vector at the patient sequence acceptor sites and co-transfecting this packaging vector with an indicator gene viral vector.

10

"Indicator or indicator gene," as described in US Patent Number 5,837,464 (International Publication Number WO 97/27319) refers to a nucleic acid encoding a protein, DNA or RNA structure that either directly or through a reaction gives rise to a measurable or noticeable aspect, e.g. a color or light of a measurable wavelength or in the case of DNA or RNA used as an indicator a change or generation of a specific DNA or RNA structure. Preferred examples of an indicator gene is the E. coli lacZ gene which encodes beta-galactosidase, the luc gene which encodes luciferase either from, for example, Photinus pyralis (the firefly) or Renilla reniformis (the sea pansy), the E. coli phoA gene which encodes alkaline phosphatase, green fluorescent protein and the bacterial CAT gene which encodes chloramphenicol acetyltransferase. The indicator or indicator gene may be functional or non-functional as described in US Patent Number 5,837,464 (International Publication Number WO 97/27319).

30 The phenotypic drug susceptibility and resistance tests of this invention may be carried out in one or more host cells as described in US Patent Number 5,837,464

(International Publication Number WO 97/27319) which is incorporated herein by reference. Viral drug susceptibility is determined as the concentration of the anti-viral agent at which a given percentage of indicator gene expression is inhibited (e.g. the IC50 for an anti-viral agent is the concentration at which 50% of indicator gene expression is inhibited). A standard curve for drug susceptibility of a given anti-viral drug can be developed for a viral segment that is either a standard laboratory viral segment or from a drug-naïve patient (i.e. a patient who has not received any anti-viral drug) using the method described in the aforementioned patent. Correspondingly, viral drug resistance is a decrease in viral drug susceptibility for a given patient compared to such a given standard or when making one or more sequential measurements in the same patient over time, as determined by decreased susceptibility in virus from later time points compared to that from earlier time points.

The antiviral drugs being added to the test system are added at selected times depending upon the target of the antiviral drug. For example, in the case of HIV protease inhibitors, including saquinavir, ritonavir, indinavir, nelfinavir and amprenavir, they are added to packaging host cells at the time of or shortly after their transfection with a resistance test vector, at an appropriate range of concentrations. HIV reverse transcriptase inhibitors, including AZT, ddI, ddC, d4T, 3TC, abacavir, nevirapine, delavirdine and efavirenz are added to target host cells at the time of or prior to infection by the resistance test vector viral particles, at an appropriate range of concentration. Alternatively,

the antiviral drugs may be present throughout the assay. The test concentration is selected from a range of concentrations which is typically between about  $8 \times 10^{-6} \mu\text{M}$  and about 2mM and more specifically for each of the following drugs: saquinavir, indinavir, nelfinavir and amprenavir, from about  $2.3 \times 10^{-5} \mu\text{M}$  to about  $1.5 \mu\text{M}$  and ritonavir, from about  $4.5 \times 10^{-5} \mu\text{M}$  to about  $3 \mu\text{M}$ .

In another embodiment of this invention, a candidate PRI antiretroviral compound is tested in the phenotypic drug susceptibility and resistance test using the resistance test vector comprising PR having a mutation at codon 88 to a serine. The candidate antiviral compound is added to the test system at an appropriate range of concentrations and at the transfection step. Alternatively, more than one candidate antiviral compound may be tested or a candidate antiviral compound may be tested in combination with an approved antiviral drug such as AZT, ddI, ddC, d4T, 3TC, abacavir, delavirdine, nevirapine, efavirenz, saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, or a compound which is undergoing clinical trials such as adefovir and ABT-378. The effectiveness of the candidate antiviral will be evaluated by measuring the expression or inhibition of the indicator gene. In another aspect of this embodiment, the drug susceptibility and resistance test may be used to screen for viral mutants. Following the identification of mutants resistant to either known antiretrovirals or candidate antiretrovirals the resistant mutants are isolated and the DNA is analyzed. A library of viral resistant mutants can thus be assembled enabling the screening of candidate PRI antiretrovirals, alone or in combination. This will enable one of ordinary skill to

identify effective PRI antiretrovirals and design effective therapeutic regimens.

5 In another embodiment of this invention, a method of assessing the effectiveness of protease antiretroviral therapy of an HIV-infected patient is provided comprising:  
(a) collecting a biological sample from the HIV-infected patient;  
(b) evaluating whether the biological sample contains  
10 nucleic acid encoding HIV protease having a mutation at codon 82 or codon 90; and  
(c) determining changes in susceptibility to protease inhibitors.

15 In another embodiment of this invention, the method is provided, wherein step (c) determines changes in susceptibility to saquinavir.

20 In another embodiment of this invention, the method is provided , wherein the mutation at codon 82 codes for alanine (A), phenylalanine (F), serine (S), or threonine (T).

25 In another embodiment of this invention, the method is provided , wherein the mutation at codon 82 is a substitution of alanine (A), phenylalanine (F), serine (S), or threonine (T) for valine(V).

30 In another embodiment of this invention, the method is provided, wherein the mutation at codon 90 codes for methionine (M).

In another embodiment of this invention, the method is provided , wherein the mutation at codon 90 is a substitution of methionine (M) for leucine (L).

5 In another embodiment of this invention, a method for evaluating the biological effectiveness of a candidate HIV protease antiretroviral drug compound is provided comprising:

10 (a) introducing a resistance test vector comprising a patient-derived segment having nucleic acid encoding HIV protease with a mutation at codon 82 or codon 90 and an indicator gene into a host cell;

(b) culturing the host cell from step (a);

15 (c) measuring the indicator gene in a target host cell; and

(d) comparing the measurement of the indicator gene from step (c) with the measurement of the indicator gene measured when steps (a) - (c) are carried out in the absence of the candidate antiretroviral drug compound;

20 wherein a test concentration of the candidate antiretroviral drug compound is present at steps (a) - (c); at steps (b) - (c); or at step (c).

25 In another embodiment of this invention, a resistance test vector comprising an HIV patient-derived segment further comprising protease having a mutation at codon 82 or codon 90 and an indicator gene, wherein the expression of the indicator gene is dependent upon the patient-derived segment.

30

In another embodiment of this invention, the resistance test vector is provided , wherein the patient-derived

segment having a mutation at codon 82 codes for alanine (A), phenylalanine (F), serine (S), or threonine (T).

5 In another embodiment of this invention, the resistance test vector of is provided , wherein the patient-derived segment having a mutation at codon 82 is a substitution of alanine (A), phenylalanine (F), serine (S), or threonine (T) for valine(V).

10 In another embodiment of this invention, the resistance test vector is provided , wherein the patient-derived segment having a mutation at codon 90 codes for methionine (M).

15 In yet another embodiment of this invention, the resistance test vector is provided, wherein the patient-derived segment having a mutation at codon 90 is a substitution of methionine (M) for leucine (L).

20 In another embodiment of this invention, a method for determining replication capacity for a patient's virus is provided comprising:

25 (a)introducing a resistance test vector comprising a patient-derived segment and an indicator gene into a host cell;

(b) culturing the host cell from (a);

(c) harvesting viral particles from step (b) and infecting target host cells;

30 (d) measuring expression of the indicator gene in the target host cell, wherein the expression of the indicator gene is dependent upon the patient-derived segment; and

(e) comparing the expression of the indicator gene from (d) with the expression of the indicator gene measured when steps (a) through (d) are carried out in a control resistance test vector.

5

In another embodiment of this invention, the method further comprises the step of:

(f) normalizing the expression of the indicator gene by measuring an amount of virus in step (c).

10

In another embodiment of this invention, the method is provided wherein the patient-derived segment comprises nucleic acid encoding HIV integrase having a mutation at codon 66.

15

In another embodiment of this invention, the method is provided wherein the patient-derived segment comprises nucleic acid encoding HIV integrase having a mutation at codon 154.

20

In another embodiment of this invention, the method is provided wherein the patient-derived segment comprises nucleic acid encoding HIV integrase having mutations at codon 66 and codon 153.

25

In another embodiment of this invention, the method is provided wherein the patient-derived segment comprises nucleic acid encoding HIV integrase having mutations at codon 66 and codon 154.

30



In another embodiment of this invention, a method is provided of assessing the effectiveness of protease antiretroviral therapy of an HIV-infected patient comprising:

- 5 (a) collecting a biological sample from the HIV-infected patient;
- (b) evaluating whether the biological sample contains nucleic acid encoding HIV protease having a mutation at codon 82 and a secondary mutation at codons selected from the group consisting of 73, 55, 48, 20, 43, 53,  
10 90, 13, 84, 23, 33, 74, 32, 39, 60, 36, and 35, or a mutation at codon 90 and a secondary mutation at codons selected from the group consisting of 53, 95, 54, 84, 82, 46, 13, 74, 55, 85, 20, 72, 62, 66, 84, 48, 33, 73, 71, 64, 93, 23, 58, and 36; and
- 15 (c) determining a change in susceptibility to a protease inhibitor.

In another embodiment of this invention, the above method is provided of assessing the effectiveness of protease  
20 antiretroviral therapy, wherein the mutation at codon 82 is a substitution of alanine (A), phenylalanine (F), serine (S), or threonine (T) for valine (V) and the mutation at codon 90 is a substitution of methionine (M) for leucine (L).

25 In another embodiment of this invention, the above method is provided of assessing the effectiveness of protease antiretroviral therapy, wherein the protease inhibitor is selected from the group consisting of indinavir, amprenavir, and saquinavir.

30

35

5 The structure, life cycle and genetic elements of the  
viruses which could be tested in the drug susceptibility  
and resistance test of this invention would be known to  
one of ordinary skill in the art. It is useful to the  
10 practice of this invention, for example, to understand the  
life cycle of a retrovirus, as well as the viral genes  
required for retrovirus rescue and infectivity.  
Retrovirally infected cells shed a membrane virus  
containing a diploid RNA genome. The virus, studded with  
an envelope glycoprotein (which serves to determine the  
15 host range of infectivity), attaches to a cellular  
receptor in the plasma membrane of the cell to be  
infected. After receptor binding, the virus is  
internalized and uncoated as it passes through the  
cytoplasm of the host cell. Either on its way to the  
20 nucleus or in the nucleus, the reverse transcriptase  
molecules resident in the viral core drive the synthesis  
of the double-stranded DNA provirus, a synthesis that is  
primed by the binding of a tRNA molecule to the genomic  
viral RNA. The double-stranded DNA provirus is  
25 subsequently integrated in the genome of the host cell,  
where it can serve as a transcriptional template for both  
mRNAs encoding viral proteins and virion genomic RNA,  
which will be packaged into viral core particles. On  
their way out of the infected cell, core particles move  
30 through the cytoplasm, attach to the inside of the plasma  
membrane of the newly infected cell, and bud, taking with  
them tracts of membrane containing the virally encoded  
envelope glycoprotein gene product. This cycle of  
infection - reverse transcription, transcription,  
35 translation, virion assembly, and budding - repeats itself

5 over and over again as infection spreads.

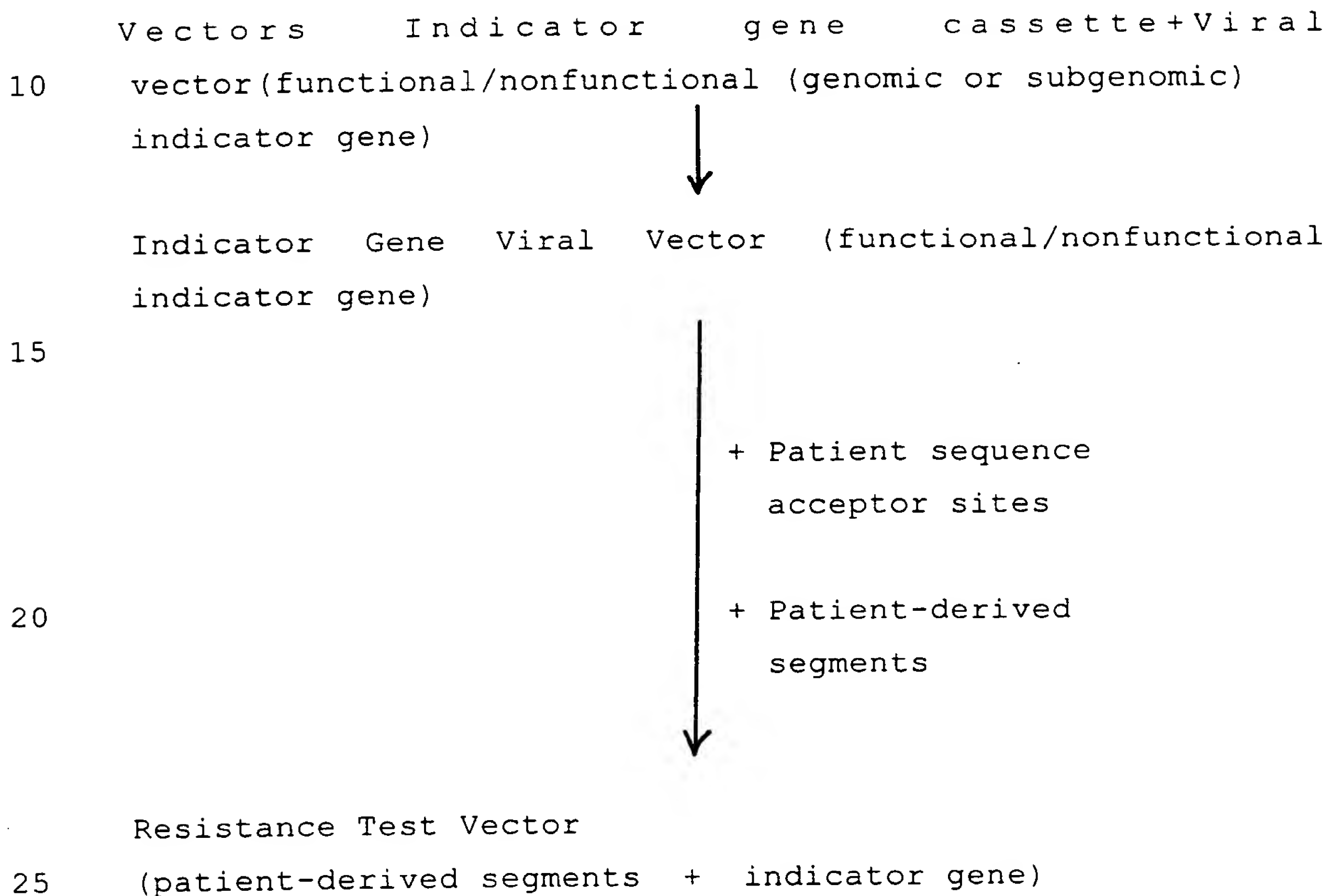
The viral RNA and, as a result, the proviral DNA encode several cis-acting elements that are vital to the successful completion of the viral lifecycle. The virion  
10 RNA carries the viral promoter at its 3' end. Replicative acrobatics place the viral promoter at the 5' end of the proviral genome as the genome is reverse transcribed. Just 3' to the 5' retroviral LTR lies the viral packaging site. The retroviral lifecycle requires the presence of  
15 virally encoded transacting factors. The viral-RNA-dependent DNA polymerase (*pol*)-reverse transcriptase is also contained within the viral core and is vital to the viral life cycle in that it is responsible for the conversion of the genomic RNA to the integrative  
20 intermediate proviral DNA. The viral envelope glycoprotein, *env*, is required for viral attachment to the uninfected cell and for viral spread. There are also transcriptional trans-activating factors, so called transactivators, that can serve to modulate the level of  
25 transcription of the integrated parental provirus. Typically, replication-competent (non-defective) viruses are self-contained in that they encode all of these trans-acting factors. Their defective counterparts are not self-contained.

30

In the case of a DNA virus, such as a hepadnavirus, understanding the life cycle and viral genes required for infection is useful to the practice of this invention. The process of HBV entry has not been well defined.  
35 Replication of HBV uses an RNA intermediate template. In the infected cell the first step in replication is the

5 conversion of the asymmetric relaxed circle DNA (rc-DNA)  
to covalently closed circle DNA (cccDNA). This process,  
which occurs within the nucleus of infected liver cells,  
involves completion of the DNA positive-strand synthesis  
and ligation of the DNA ends. In the second step, the  
10 cccDNA is transcribed by the host RNA polymerase to  
generate a 3.5 kB RNA template (the pregenome). This  
pregenome is complexed with protein in the viral core.  
The third step involves the synthesis of the first  
negative-sense DNA strand by copying the pregenomic RNA  
15 using the virally encoded P protein reverse transcriptase.  
The P protein also serves as the minus strand DNA primer.  
Finally, the synthesis of the second positive-sense DNA  
strand occurs by copying the first DNA strand, using the P  
protein DNA polymerase activity and an oligomer of viral  
20 RNA as primer. The pregenome also transcribes mRNA for  
the major structural core proteins.

5 The following flow chart illustrates certain of the various vectors and host cells which may be used in this invention. It is not intended to be all inclusive.



#### Host Cells

30 Packaging Host Cell - transfected with packaging  
expression vectors

Resistance Test Vector Host Cell - a packaging host cell  
transfected with a resistance test vector

35 Target Host Cell - a host cell to be infected by a

5 resistance test vector viral particle produced by the  
resistance test vector host cell

**Resistance Test Vector**

10 "Resistance test vector" means one or more vectors which  
taken together contain DNA or RNA comprising a  
patient-derived segment and an indicator gene. In the  
case where the resistance test vector comprises more than  
one vector the patient-derived segment may be contained in  
one vector and the indicator gene in a different vector.  
15 Such a resistance test vector comprising more than one  
vector is referred to herein as a resistance test vector  
system for purposes of clarity but is nevertheless  
understood to be a resistance test vector. The DNA or RNA  
of a resistance test vector may thus be contained in one  
20 or more DNA or RNA molecules. In one embodiment, the  
resistance test vector is made by insertion of a  
patient-derived segment into an indicator gene viral  
vector. In another embodiment, the resistance test vector  
is made by insertion of a patient-derived segment into a  
25 packaging vector while the indicator gene is contained in  
a second vector, for example an indicator gene viral  
vector. As used herein, "patient-derived segment" refers  
to one or more viral segments obtained directly from a  
patient using various means, for example, molecular  
30 cloning or polymerase chain reaction (PCR) amplification  
of a population of patient-derived segments using viral  
DNA or complementary DNA (cDNA) prepared from viral RNA,  
present in the cells (e.g. peripheral blood mononuclear  
cells, PBMC), serum or other bodily fluids of infected  
35 patients. When a viral segment is "obtained directly"  
from a patient it is obtained without passage of the virus

5 through culture, or if the virus is cultured, then by a  
minimum number of passages to essentially eliminate the  
selection of mutations in culture. The term "viral  
segment" refers to any functional viral sequence or viral  
gene encoding a gene product (e.g., a protein) that is the  
10 target of an anti-viral drug. The term "functional viral  
sequence" as used herein refers to any nucleic acid  
sequence (DNA or RNA) with functional activity such as  
enhancers, promoters, polyadenylation sites, sites of  
action of trans-acting factors, such as *tar* and RRE,  
15 packaging sequences, integration sequences, or splicing  
sequences. If a drug were to target more than one  
functional viral sequence or viral gene product then  
patient-derived segments corresponding to each said viral  
gene would be inserted in the resistance test vector. In  
20 the case of combination therapy where two or more  
anti-virals targeting two different functional viral  
sequences or viral gene products are being evaluated,  
patient-derived segments corresponding to each functional  
viral sequence or viral gene product would be inserted in  
25 the resistance test vector. The patient-derived segments  
are inserted into unique restriction sites or specified  
locations, called patient sequence acceptor sites, in the  
indicator gene viral vector or for example, a packaging  
vector depending on the particular construction being used  
30 as described herein.

As used herein, "patient-derived segment" encompasses  
segments derived from human and various animal species.  
Such species include, but are not limited to chimpanzees,  
35 horses, cattles, cats and dogs.

5 Patient-derived segments can also be incorporated into  
resistance test vectors using any of several alternative  
cloning techniques. For example, cloning via the  
introduction of class II restriction sites into both the  
plasmid backbone and the patient-derived segments or by  
10 uracil DNA glycosylase primer cloning (refs).

The patient-derived segment may be obtained by any method  
of molecular cloning or gene amplification, or  
modifications thereof, by introducing patient sequence  
15 acceptor sites, as described below, at the ends of the  
patient-derived segment to be introduced into the  
resistance test vector. For example, in a gene  
amplification method such as PCR, restriction sites  
corresponding to the patient-sequence acceptor sites can  
20 be incorporated at the ends of the primers used in the PCR  
reaction. Similarly, in a molecular cloning method such  
as cDNA cloning, said restriction sites can be  
incorporated at the ends of the primers used for first or  
second strand cDNA synthesis, or in a method such as  
25 primer-repair of DNA, whether cloned or uncloned DNA, said  
restriction sites can be incorporated into the primers  
used for the repair reaction. The patient sequence  
acceptor sites and primers are designed to improve the  
representation of patient-derived segments. Sets of  
30 resistance test vectors having designed patient sequence  
acceptor sites provide representation of patient-derived  
segments that would be underrepresented in one resistance  
test vector alone.

35 Resistance test vectors are prepared by modifying an  
indicator gene viral vector (described below) by



5     introducing patient sequence acceptor sites, amplifying or  
cloning patient-derived segments and inserting the  
amplified or cloned sequences precisely into indicator  
gene viral vectors at the patient sequence acceptor sites.

10     The resistance test vectors are constructed from  
indicator gene viral vectors which are in turn derived  
from genomic viral vectors or subgenomic viral vectors and  
an indicator gene cassette, each of which is described  
below. Resistance test vectors are then introduced into a  
host cell. Alternatively, a resistance test vector (also  
15     referred to as a resistance test vector system) is  
prepared by introducing patient sequence acceptor sites  
into a packaging vector, amplifying or cloning  
patient-derived segments and inserting the amplified or  
cloned sequences precisely into the packaging vector at  
20     the patient sequence acceptor sites and co-transfecting  
this packaging vector with an indicator gene viral vector.

In one preferred embodiment, the resistance test vector  
may be introduced into packaging host cells together with  
25     packaging expression vectors, as defined below, to produce  
resistance test vector viral particles that are used in  
drug resistance and susceptibility tests that are referred  
to herein as a "particle-based test." In an alternative  
preferred embodiment, the resistance test vector may be  
30     introduced into a host cell in the absence of packaging  
expression vectors to carry out a drug resistance and  
susceptibility test that is referred to herein as a  
"non-particle-based test." As used herein a "packaging  
expression vector" provides the factors, such as packaging  
35     proteins (e.g. structural proteins such as core and  
envelope polypeptides), transacting factors, or genes

5 required by replication-defective retrovirus or  
hepadnavirus. In such a situation, a  
replication-competent viral genome is enfeebled in a  
manner such that it cannot replicate on its own. This  
means that, although the packaging expression vector can  
10 produce the trans-acting or missing genes required to  
rescue a defective viral genome present in a cell  
containing the enfeebled genome, the enfeebled genome  
cannot rescue itself.

15 ***Indicator or Indicator Gene***

"Indicator or indicator gene" refers to a nucleic acid  
encoding a protein, DNA or RNA structure that either  
directly or through a reaction gives rise to a measurable  
or noticeable aspect, e.g. a color or light of a  
20 measurable wavelength or in the case of DNA or RNA used as  
an indicator a change or generation of a specific DNA or  
RNA structure. Preferred examples of an indicator gene is  
the *E. coli lacZ* gene which encodes beta-galactosidase,  
the *luc* gene which encodes luciferase either from, for  
25 example, *Photinus pyralis* (the firefly) or *Renilla*  
*reniformis* (the sea pansy), the *E. coli phoA* gene which  
encodes alkaline phosphatase, green fluorescent protein  
and the bacterial CAT gene which encodes chloramphenicol  
acetyltransferase. Additional preferred examples of an  
30 indicator gene are secreted proteins or cell surface  
proteins that are readily measured by assay, such as  
radioimmunoassay (RIA), or fluorescent activated cell  
sorting (FACS), including, for example, growth factors,  
cytokines and cell surface antigens (e.g. growth hormone,  
35 Il-2 or CD4, respectively). "Indicator gene" is  
understood to also include a selection gene, also referred

5 to as a selectable marker. Examples of suitable  
selectable markers for mammalian cells are dihydrofolate  
reductase (DHFR), thymidine kinase, hygromycin, neomycin,  
zeocin or *E. coli* gpt. In the case of the foregoing  
10 examples of indicator genes, the indicator gene and the  
patient-derived segment are discrete, i.e. distinct and  
separate genes. In some cases a patient-derived segment  
may also be used as an indicator gene. In one such  
embodiment in which the patient-derived segment  
15 corresponds to more than one viral gene which is the  
target of an anti-viral, one of said viral genes may also  
serve as the indicator gene. For example, a viral  
protease gene may serve as an indicator gene by virtue of  
its ability to cleave a chromogenic substrate or its  
20 ability to activate an inactive zymogen which in turn  
cleaves a chromogenic substrate, giving rise in each case  
to a color reaction. In all of the above examples of  
indicator genes, the indicator gene may be either  
"functional" or "non-functional" but in each case the  
25 expression of the indicator gene in the target cell is  
ultimately dependent upon the action of the  
patient-derived segment.

#### Functional Indicator Gene

In the case of a "functional indicator gene" the indicator  
30 gene may be capable of being expressed in a "packaging  
host cell/resistance test vector host cell" as defined  
below, independent of the patient-derived segment,  
however the functional indicator gene could not be  
expressed in the target host cell, as defined below,  
35 without the production of functional resistance test  
vector particles and their effective infection of the

5 target host cell. In one embodiment of a functional  
indicator gene, the indicator gene cassette, comprising  
control elements and a gene encoding an indicator protein,  
is inserted into the indicator gene viral vector with the  
same or opposite transcriptional orientation as the native  
10 or foreign enhancer/promoter of the viral vector. One  
example of a functional indicator gene in the case of HIV  
or HBV, places the indicator gene and its promoter (a CMV  
IE enhancer/promoter) in the same or opposite  
transcriptional orientation as the HIV-LTR or HBV  
15 enhancer-promoter, respectively, or the CMV IE  
enhancer/promoter associated with the viral vector.

#### Non-Functional Indicator Gene

Alternatively the indicator gene, may be "non-functional"  
20 in that the indicator gene is not efficiently expressed in  
a packaging host cell transfected with the resistance test  
vector, which is then referred to a resistance test vector  
host cell, until it is converted into a functional  
indicator gene through the action of one or more of the  
25 patient-derived segment products. An indicator gene is  
rendered non-functional through genetic manipulation  
according to this invention.

1. Permuted Promoter In one embodiment an indicator gene  
30 is rendered non-functional due to the location of the  
promoter, in that, although the promoter is in the same  
transcriptional orientation as the indicator gene, it  
follows rather than precedes the indicator gene coding  
sequence. This misplaced promoter is referred to as a  
35 "permuted promoter." In addition to the permuted promoter  
the orientation of the non-functional indicator gene is

5        opposite to that of the native or foreign  
promoter/enhancer of the viral vector. Thus the coding  
sequence of the non-functional indicator gene can neither  
be transcribed by the permuted promoter nor by the viral  
promoters. The non-functional indicator gene and its  
10       permuted promoter is rendered functional by the action of  
one or more of the viral proteins. One example of a  
non-functional indicator gene with a permuted promoter in  
the case of HIV, places a T7 phage RNA polymerase promoter  
(herein referred to as T7 promoter) promoter in the 5' LTR  
15       in the same transcriptional orientation as the indicator  
gene. The indicator gene cannot be transcribed by the T7  
promoter as the indicator gene cassette is positioned  
upstream of the T7 promoter. The non-functional indicator  
gene in the resistance test vector is converted into a  
20       functional indicator gene by reverse transcriptase upon  
infection of the target cells, resulting from the  
repositioning of the T7 promoter, by copying from the 5'  
LTR to the 3' LTR, relative to the indicator gene coding  
region. Following the integration of the repaired  
25       indicator gene into the target cell chromosome by HIV  
integrase, a nuclear T7 RNA polymerase expressed by the  
target cell transcribes the indicator gene. One example  
of a non-functional indicator gene with a permuted  
promoter in the case of HBV, places an enhancer-promoter  
30       region downstream or 3' of the indicator gene both having  
the same transcriptional orientation. The indicator gene  
cannot be transcribed by the enhancer-promoter as the  
indicator gene cassette is positioned upstream. The  
non-functional indicator gene in the resistance test  
35       vector is converted into a functional indicator gene by  
reverse transcription and circularization of the HBV

5 indicator gene viral vector by the repositioning of the  
enhancer-promoter upstream relative to the indicator gene  
coding region.

10 A permuted promoter may be any eukaryotic or prokaryotic  
promoter which can be transcribed in the target host cell.  
Preferably the promoter will be small in size to enable  
insertion in the viral genome without disturbing viral  
replication. More preferably, a promoter that is small in  
size and is capable of transcription by a single subunit  
15 RNA polymerase introduced into the target host cell, such  
as a bacteriophage promoter, will be used. Examples of  
such bacteriophage promoters and their cognate RNA  
polymerases include those of phages T7, T3 and Sp6. A  
nuclear localization sequence (NLS) may be attached to the  
20 RNA polymerase to localize expression of the RNA  
polymerase to the nucleus where they may be needed to  
transcribed the repaired indicator gene. Such an NLS may  
be obtained from any nuclear-transported protein such as  
the SV40 T antigen. If a phage RNA polymerase is  
25 employed, an internal ribosome entry site (IRES) such as  
the EMC virus 5' untranslated region (UTR) may be added in  
front of the indicator gene, for translation of the  
transcripts which are generally uncapped. In the case of  
HIV, the permuted promoter itself can be introduced at any  
30 position within the 5' LTR that is copied to the 3' LTR  
during reverse transcription so long as LTR function is  
not disrupted, preferably within the U5 and R portions of  
the LTR, and most preferably outside of functionally  
important and highly conserved regions of U5 and R. In  
35 the case of HBV, the permuted promoter can be placed at  
any position that does not disrupt the cis acting elements

5 that are necessary for HBV DNA replication. Blocking  
sequences may be added at the ends of the resistance test  
vector should there be inappropriate expression of the  
non-functional indicator gene due to transfection  
artifacts (DNA concatenation). In the HIV example of the  
10 permuted T7 promoter given above, such a blocking sequence  
may consist of a T7 transcriptional terminator, positioned  
to block readthrough transcription resulting from DNA  
concatenation, but not transcription resulting from  
repositioning of the permuted T7 promoter from the 5' LTR  
15 to the 3' LTR during reverse transcription.

2. Permuted Coding Region In a second embodiment, an  
indicator gene is rendered non-functional due to the  
relative location of the 5' and 3' coding regions of the  
20 indicator gene, in that, the 3' coding region precedes  
rather than follows the 5' coding region. This misplaced  
coding region is referred to as a "permuted coding  
region." The orientation of the non-functional indicator  
gene may be the same or opposite to that of the native or  
25 foreign promoter/enhancer of the viral vector, as mRNA  
coding for a functional indicator gene will be produced in  
the event of either orientation. The non-functional  
indicator gene and its permuted coding region is rendered  
functional by the action of one or more of the  
30 patient-derived segment products. A second example of a  
non-functional indicator gene with a permuted coding  
region in the case of HIV, places a 5' indicator gene  
coding region with an associated promoter in the 3' LTR U3  
region and a 3' indicator gene coding region in an  
35 upstream location of the HIV genome, with each coding  
region having the same transcriptional orientation as the



5 viral LTRs. In both examples, the 5' and 3' coding  
regions may also have associated splice donor and acceptor  
sequences, respectively, which may be heterologous or  
artificial splicing signals. The indicator gene cannot be  
functionally transcribed either by the associated promoter  
10 or viral promoters, as the permuted coding region prevents  
the formation of functionally spliced transcripts. The  
non-functional indicator gene in the resistance test  
vector is converted into a functional indicator gene by  
reverse transcriptase upon infection of the target cells,  
15 resulting from the repositioning of the 5' and 3'  
indicator gene coding regions relative to one another, by  
copying of the 3' LTR to the 5' LTR. Following  
transcription by the promoter associated with the 5'  
coding region, RNA splicing can join the 5' and 3' coding  
20 regions to produce a functional indicator gene product.  
One example of a non-functional indicator gene with a  
permuted coding region in the case of HBV, places a 3'  
indicator gene coding region upstream or 5' of the  
enhancer-promoter and the 5' coding region of the  
25 indicator gene. The transcriptional orientation of the  
indicator gene 5' and 3' coding regions are identical to  
one another, and the same as that of the indicator gene  
viral vector. However, as the indicator gene 5' and 3'  
coding regions are permuted in the resistance test vectors  
30 (i.e., the 5' coding region is downstream of the 3' coding  
region), no mRNA is transcribed which can be spliced to  
generate a functional indicator gene coding region.  
Following reverse transcription and circularization of the  
indicator gene viral vector, the indicator gene 3' coding  
35 region is positioned downstream or 3' to the  
enhancer-promoter and 5' coding regions thus permitting



5 the transcription of mRNA which can be spliced to generate  
a functional indicator gene coding region.

3. Inverted Intron In a third embodiment, the indicator  
gene is rendered non-functional through use of an  
10 "inverted intron," i.e. an intron inserted into the coding  
sequence of the indicator gene with a transcriptional  
orientation opposite to that of the indicator gene. The  
overall transcriptional orientation of the indicator gene  
cassette including its own, linked promoter, is opposite  
15 to that of the viral control elements, while the  
orientation of the artificial intron is the same as the  
viral control elements. Transcription of the indicator  
gene by its own linked promoter does not lead to the  
production of functional transcripts as the inverted  
20 intron cannot be spliced in this orientation.  
Transcription of the indicator gene by the viral control  
elements does, however, lead to the removal of the  
inverted intron by RNA splicing, although the indicator  
gene is still not functionally expressed as the resulting  
25 transcript has an antisense orientation. Following the  
reverse transcription of this transcript and integration  
of the resultant retroviral DNA, or the circularization of  
hepadnavirus DNA, the indicator gene can be functionally  
transcribed using its own linked promoter as the inverted  
30 intron has been previously removed. In this case, the  
indicator gene itself may contain its own functional  
promoter with the entire transcriptional unit oriented  
opposite to the viral control elements. Thus the  
non-functional indicator gene is in the wrong orientation  
35 to be transcribed by the viral control elements and it  
cannot be functionally transcribed by its own promoter, as

5 the inverted intron cannot be properly excised by  
splicing. However, in the case of a retrovirus and HIV  
specifically and hepadnaviruses, and HBV specifically,  
transcription by the viral promoters (HIV LTR or HBV  
enhancer-promoter) results in the removal of the inverted  
10 intron by splicing. As a consequence of reverse  
transcription of the resulting spliced transcript and the  
integration of the resulting provirus into the host cell  
chromosome or circularization of the HBV vector, the  
indicator gene can now be functionally transcribed by its  
15 own promoter. The inverted intron, consisting of a splice  
donor and acceptor site to remove the intron, is  
preferably located in the coding region of the indicator  
gene in order to disrupt translation of the indicator  
gene. The splice donor and acceptor may be any splice  
20 donor and acceptor. A preferred splice donor-receptor is  
the CMV IE splice donor and the splice acceptor of the  
second exon of the human alpha globin gene ("intron A").

#### ***Indicator Gene Viral Vector - Construction***

25 As used herein, "indicator gene viral vector" refers to a  
vector(s) comprising an indicator gene and its control  
elements and one or more viral genes. The indicator gene  
viral vector is assembled from an indicator gene cassette  
and a "viral vector," defined below. The indicator gene  
30 viral vector may additionally include an enhancer,  
splicing signals, polyadenylation sequences,  
transcriptional terminators, or other regulatory  
sequences. Additionally the indicator gene viral vector  
may be functional or nonfunctional. In the event that the  
35 viral segments which are the target of the anti-viral drug  
are not included in the indicator gene viral vector they

5 are provided in a second vector. An "indicator gene cassette" comprises an indicator gene and control elements. "Viral vector" refers to a vector comprising some or all of the following: viral genes encoding a gene product, control sequences, viral packaging sequences, and  
10 in the case of a retrovirus, integration sequences. The viral vector may additionally include one or more viral segments one or more of which may be the target of an anti-viral drug. Two examples of a viral vector which contain viral genes are referred to herein as an "genomic viral vector" and a "subgenomic viral vector." A "genomic  
15 viral vector" is a vector which may comprise a deletion of a one or more viral genes to render the virus replication incompetent, but which otherwise preserves the mRNA expression and processing characteristics of the complete virus. In one embodiment for an HIV drug susceptibility and resistance test, the genomic viral vector comprises the HIV *gag-pol*, *vif*, *vpr*, *tat*, *rev*, *vpu*, and *nef* genes (some, most or all of *env* may be deleted). A "subgenomic  
20 viral vector" refers to a vector comprising the coding region of one or more viral genes which may encode the proteins that are the target(s) of the anti-viral drug. In the case of HIV, a preferred embodiment is a subgenomic viral vector comprising the HIV *gag-pol* gene. In the case of HBV a preferred embodiment is a subgenomic viral vector comprising the HBV P gene. In the case of HIV, two  
25 examples of proviral clones used for viral vector construction are: HXB2 (Fisher et al., (1986) *Nature*, **320**, 367-371) and NL4-3, (Adachi et al., (1986) *J. Virol.*, **59**, 284-291). In the case of HBV, a large number of full  
30 length genomic sequences have been characterized and could be used for construction of HBV viral vectors: GenBank

35

5 Nos. M54923, M38636, J02203 and X59795. The viral coding  
genes may be under the control of a native  
enhancer/promoter or a foreign viral or cellular  
enhancer/promoter. A preferred embodiment for an HIV drug  
susceptibility and resistance test, is to place the  
10 genomic or subgenomic viral coding regions under the  
control of the native enhancer/promoter of the HIV-LTR U3  
region or the CMV immediate-early (IE) enhancer/promoter.  
A preferred embodiment for an HBV drug susceptibility and  
resistance test, is to place the genomic or subgenomic  
15 viral coding regions under the control of the CMV  
immediate-early (IE) enhancer/promoter. In the case of an  
indicator gene viral vector that contains one or more  
viral genes which are the targets or encode proteins which  
are the targets of an anti-viral drug(s) then said vector  
20 contains the patient sequence acceptor sites. The  
patient-derived segments are inserted in the patient  
sequence acceptor site in the indicator gene viral vector  
which is then referred to as the resistance test vector,  
as described above.

25 "Patient sequence acceptor sites" are sites in a vector  
for insertion of patient-derived segments and said sites  
may be: 1) unique restriction sites introduced by  
site-directed mutagenesis into a vector; 2) naturally  
30 occurring unique restriction sites in the vector; or 3)  
selected sites into which a patient-derived segment may be  
inserted using alternative cloning methods (e.g. UDG  
cloning). In one embodiment the patient sequence acceptor  
site is introduced into the indicator gene viral vector.  
35 The patient sequence acceptor sites are preferably located  
within or near the coding region of the viral protein

5 which is the target of the anti-viral drug. The viral  
sequences used for the introduction of patient sequence  
acceptor sites are preferably chosen so that no change, or  
a conservative change, is made in the amino acid coding  
sequence found at that position. Preferably the patient  
10 sequence acceptor sites are located within a relatively  
conserved region of the viral genome to facilitate  
introduction of the patient-derived segments.  
Alternatively, the patient sequence acceptor sites are  
located between functionally important genes or regulatory  
15 sequences. Patient-sequence acceptor sites may be located  
at or near regions in the viral genome that are relatively  
conserved to permit priming by the primer used to  
introduce the corresponding restriction site into the  
patient-derived segment. To improve the representation of  
20 patient-derived segments further, such primers may be  
designed as degenerate pools to accommodate viral sequence  
heterogeneity, or may incorporate residues such as  
deoxyinosine (I) which have multiple base-pairing  
capabilities. Sets of resistance test vectors having  
25 patient sequence acceptor sites that define the same or  
overlapping restriction site intervals may be used  
together in the drug resistance and susceptibility tests  
to provide representation of patient-derived segments that  
contain internal restriction sites identical to a given  
30 patient sequence acceptor site, and would thus be  
underrepresented in either resistance test vector alone.

#### **Host Cells**

The resistance test vector is introduced into a host cell.  
35 Suitable host cells are mammalian cells. Preferred host  
cells are derived from human tissues and cells which are

5 the principle targets of viral infection. In the case of  
HIV these include human cells such as human T cells,  
monocytes, macrophage, dendritic cells, Langerhans cells,  
hematopoietic stem cells or precursor cells, and other  
cells. In the case of HBV, suitable host cells include  
10 hepatoma cell lines (HepG2, Huh 7), primary human  
hepatocytes, mammalian cells which can be- infected by  
pseudotyped HBV, and other cells. Human derived host  
cells will assure that the anti-viral drug will enter the  
cell efficiently and be converted by the cellular  
15 enzymatic machinery into the metabolically relevant form  
of the anti-viral inhibitor. Host cells are referred to  
herein as a "packaging host cells," "resistance test  
vector host cells," or "target host cells." A "packaging  
host cell" refers to a host cell that provides the  
20 trans-acting factors and viral packaging proteins required  
by the replication defective viral vectors used herein,  
such as the resistance test vectors, to produce resistance  
test vector viral particles. The packaging proteins may  
be provided for by the expression of viral genes contained  
25 within the resistance test vector itself, a packaging  
expression vector(s), or both. A packaging host cell is a  
host cell which is transfected with one or more packaging  
expression vectors and when transfected with a resistance  
test vector is then referred to herein as a "resistance  
30 test vector host cell" and is sometimes referred to as a  
packaging host cell/resistance test vector host cell.  
Preferred host cells for use as packaging host cells for  
HIV include 293 human embryonic kidney cells (293, Graham,  
F.L. et al., J. Gen Virol. 36: 59, 1977), BOSC23 (Pear et  
al., Proc. Natl. Acad. Sci. 90, 8392, 1993), tsa54 and  
35 tsa201 cell lines (Heinzel et al., J.Virol. 62,

5 3738,1988), for HBV HepG2 (Galle and Theilmann, L.  
Arzheim.-Forschy *Drug Res.* (1990) **40**, 1380-1382). (Huh,  
Ueda, K et al. *Virology* \*1989) **169**, 213-216). A "target  
10 test vector viral particles produced by the resistance  
test vector host cell in which expression or inhibition of  
the indicator gene takes place. Preferred host cells for  
use as target host cells include human T cell leukemia  
cell lines including Jurkat (ATCC T1B-152), H9 (ATCC  
HTB-176), CEM (ATCC CCL-119), HUT78 (ATCC T1B-161), and  
15 derivatives thereof.

This invention is illustrated in the Experimental Details  
section which follows. These sections are set forth to  
aid in an understanding of the invention but are not  
20 intended to, and should not be construed to, limit in any  
way the invention as set forth in the claims which follow  
thereafter.

### Experimental Details

#### 25 General Materials and Methods

Most of the techniques used to construct vectors, and  
transfect and infect cells, are widely practiced in the  
art, and most practitioners are familiar with the standard  
resource materials that describe specific conditions and  
30 procedures. However, for convenience, the following  
paragraphs may serve as a guideline.

As used herein, "replication capacity" is defined herein  
is a measure of how well the virus replicates. This may  
35 also be referred to as viral fitness. In one embodiment,  
replication capacity can be measured by evaluating the



5 ability of the virus to replicate in a single round of replication.

As used herein, "control resistance test vector" is defined as a resistance test vector comprising a standard  
10 viral sequence (for example, HXB2, PNL4-3) and an indicator gene.

As used herein, "normalizing" is defined as standardizing the amount of the expression of indicator gene measured  
15 relative to the number of viral particles giving rise to the expression of the indicator gene. For example, normalization is measured by dividing the amount of luciferase activity measured by the number of viral particles measured at the time of infection.

20 "Plasmids" and "vectors" are designated by a lower case p followed by letters and/or numbers. The starting plasmids herein are either commercially available, publicly available on an unrestricted basis, or can be constructed  
25 from available plasmids in accord with published procedures. In addition, equivalent plasmids to those described are known in the art and will be apparent to the ordinarily skilled artisan.

30 Construction of the vectors of the invention employs standard ligation and restriction techniques which are well understood in the art (see Ausubel et al., (1987) Current Protocols in Molecular Biology, Wiley -  
Interscience or Maniatis et al., (1992) in Molecular  
35 Cloning: A laboratory Manual, Cold Spring Harbor Laboratory, N.Y.). Isolated plasmids, DNA sequences, or



5 synthesized oligonucleotides are cleaved, tailored, and  
religated in the form desired. The sequences of all DNA  
constructs incorporating synthetic DNA were confirmed by  
DNA sequence analysis (Sanger et al. (1977) Proc. Natl.  
Acad. Sci. 74, 5463-5467).

10

"Digestion" of DNA refers to catalytic cleavage of the DNA  
with a restriction enzyme that acts only at certain  
sequences, restriction sites, in the DNA. The various  
restriction enzymes used herein are commercially available  
15 and their reaction conditions, cofactors and other  
requirements are known to the ordinarily skilled artisan.  
For analytical purposes, typically 1 µg of plasmid or DNA  
fragment is used with about 2 units of enzyme in about 20  
µl of buffer solution. Alternatively, an excess of  
20 restriction enzyme is used to insure complete digestion of  
the DNA substrate. Incubation times of about one hour to  
two hours at about 37°C are workable, although variations  
can be tolerated. After each incubation, protein is  
removed by extraction with phenol/chloroform and the  
25 nucleic acid recovered from aqueous fractions by  
precipitation with ethanol. If desired, size separation  
of the cleaved fragments may be performed by  
polyacrylamide gel or agarose gel electrophoresis using  
standard techniques. A general description of size  
30 separations is found in Methods of Enzymology 65:499-560  
(1980).

Restriction cleaved fragments may be blunt ended by  
treating with the large fragment of E. coli DNA polymerase  
35 I (Klenow) in the presence of the four deoxynucleotide  
triphosphates (dNTPs) using incubation times of about 15

5 to 25 minutes at 20°C in 50 mM Tris (pH 7.6) 50 mM NaCl, 6  
mM MgCl<sub>2</sub>, 6 mM DTT and 5-10 mM dNTPs. The Klenow fragment  
fills in at 5' sticky ends but chews back protruding 3'  
single strands, even though the four dNTPs are present.  
If desired, selective repair can be performed by supplying  
10 only one of the dNTPs, or with selected dNTPs, within the  
limitations dictated by the nature of the sticky ends.  
After treatment with Klenow, the mixture is extracted with  
phenol/chloroform and ethanol precipitated. Treatment  
under appropriate conditions with S1 nuclease or Bal-31  
15 results in hydrolysis of any single-stranded portion.

Ligations are performed in 15-50 µl volumes under the  
following standard conditions and temperatures: 20 mM  
Tris-Cl pH 7.5, 10 mM MgCl<sub>2</sub>, 10 mM DTT, 33 mg/ml BSA, 10  
20 mM- 50 mM NaCl, and either 40 µM ATP, 0.01-0.02 (Weiss)  
units T4 DNA ligase at 0°C (for "sticky end" ligation) or  
1mM ATP, 0.3 - 0.6 (Weiss) units T4 DNA ligase at 14°C (for  
"blunt end" ligation). Intermolecular "sticky end"  
ligations are usually performed at 33-100 µg/ml total DNA  
25 concentrations (5-100 mM total end concentration).  
Intermolecular blunt end ligations (usually employing a  
10-30 fold molar excess of linkers) are performed at 1µM  
total ends concentration.

30 "Transient expression" refers to unamplified expression  
within about one day to two weeks of transfection. The  
optimal time for transient expression of a particular  
desired heterologous gene may vary depending on several  
factors including, for example, any transacting factors  
35 which may be employed, translational control mechanisms  
and the host cell. Transient expression occurs when the

5 particular plasmid that has been transfected functions,  
i.e., is transcribed and translated. During this time the  
plasmid DNA which has entered the cell is transferred to  
the nucleus. The DNA is in a nonintegrated state, free  
within the nucleus. Transcription of the plasmid taken up  
10 by the cell occurs during this period. Following  
transfection the plasmid DNA may become degraded or  
diluted by cell division. Random integration within the  
cell chromatin occurs.

15 In general, vectors containing promoters and control  
sequences which are derived from species compatible with  
the host cell are used with the particular host cell.  
Promoters suitable for use with prokaryotic hosts  
illustratively include the beta-lactamase and lactose  
20 promoter systems, alkaline phosphatase, the tryptophan  
(trp) promoter system and hybrid promoters such as tac  
promoter. However, other functional bacterial promoters  
are suitable. In addition to prokaryotes, eukaryotic  
microbes such as yeast cultures may also be used.  
25 *Saccharomyces cerevisiae*, or common baker's yeast is the  
most commonly used eukaryotic microorganism, although a  
number of other strains are commonly available. Promoters  
controlling transcription from vectors in mammalian host  
cells may be obtained from various sources, for example,  
30 the genomes of viruses such as: polyoma, simian virus 40  
(SV40), adenovirus, retroviruses, hepatitis B virus and  
preferably cytomegalovirus, or from heterologous mammalian  
promoters, e.g.  $\beta$ -actin promoter. The early and late  
promoters of the SV 40 virus are conveniently obtained as  
35 an SV40 restriction fragment that also contains the SV40  
viral origin of replication. The immediate early promoter

5 of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment. Of course, promoters from the host cell or related species also are useful herein.

10 The vectors used herein may contain a selection gene, also termed a selectable marker. A selection gene encodes a protein, necessary for the survival or growth of a host cell transformed with the vector. Examples of suitable  
15 selectable markers for mammalian cells include the dihydrofolate reductase gene (DHFR), the ornithine decarboxylase gene, the multi-drug resistance gene (mdr), the adenosine deaminase gene, and the glutamine synthase gene. When such selectable markers are successfully transferred into a mammalian host cell, the transformed mammalian host cell can survive if placed under selective  
20 pressure. There are two widely used distinct categories of selective regimes. The first category is based on a cell's metabolism and the use of a mutant cell line which lacks the ability to grow independent of a supplemented media. The second category is referred to as dominant  
25 selection which refers to a selection scheme used in any cell type and does not require the use of a mutant cell line. These schemes typically use a drug to arrest growth of a host cell. Those cells which have a novel gene would express a protein conveying drug resistance and would  
30 survive the selection. Examples of such dominant selection use the drugs neomycin (Southern and Berg (1982) J. Molec. Appl. Genet. 1, 327), mycophenolic acid (Mulligan and Berg (1980) Science 209, 1422), or hygromycin (Sugden et al. (1985) Mol. Cell. Biol. 5,  
35 410-413). The three examples given above employ bacterial genes under eukaryotic control to convey resistance to the

5 appropriate drug neomycin (G418 or gentamicin), xgpt  
(mycophenolic acid) or hygromycin, respectively.

10 "Transfection" means introducing DNA into a host cell so  
that the DNA is expressed, whether functionally expressed  
or otherwise; the DNA may also replicate either as an  
extrachromosomal element or by chromosomal integration.  
Unless otherwise provided, the method used herein for  
transfection of the host cells is the calcium phosphate  
co-precipitation method of Graham and van der Eb (1973)  
15 Virology 52, 456-457. Alternative methods for  
transfection are electroporation, the DEAE-dextran method,  
lipofection and biolistics (Kriegler (1990) Gene Transfer  
and Expression: A Laboratory Manual, Stockton Press).

20 Host cells may be transfected with the expression vectors  
of the present invention and cultured in conventional  
nutrient media modified as is appropriate for inducing  
promoters, selecting transformants or amplifying genes.  
Host cells are cultured in F12:DMEM (Gibco) 50:50 with  
25 added glutamine. The culture conditions, such as  
temperature, pH and the like, are those previously used  
with the host cell selected for expression, and will be  
apparent to the ordinarily skilled artisan.

30 The following examples merely illustrate the best mode now  
known for practicing the invention, but should not be  
construed to limit the invention. All publications and  
patent applications cited in this specification are herein  
incorporated by reference in their entirety as if each  
35 individual publication or patent application were  
specifically and individually indicated to be incorporated

5 by reference.

# **EXAMPLE 1**

## **Phenotypic Drug Susceptibility and Resistance Test Using Resistance Test Vectors**

10 Phenotypic drug susceptibility and resistance tests are carried out using the means and methods described in US Patent Number 5,837,464 (International Publication Number WO 97/27319) which is hereby incorporated by reference.

15 In these experiments patient-derived segment(s) corresponding to the HIV protease and reverse transcriptase coding regions were either patient-derived segments amplified by the reverse transcription-polymerase chain reaction method (RT-PCR) using viral RNA isolated

20 from viral particles present in the serum of HIV-infected individuals or were mutants of wild type HIV-1 made by site directed mutagenesis of a parental clone of resistance test vector DNA. Isolation of viral RNA was performed using standard procedures (e.g. RNeasy Total

25 RNA Isolation System, Promega, Madison WI or RNeasy, Tel-Test, Friendswood, TX). The RT-PCR protocol was divided into two steps. A retroviral reverse transcriptase [e.g. Moloney MuLV reverse transcriptase (Roche Molecular Systems, Inc., Branchburg, NJ), or avian

30 myeloblastosis virus (AMV) reverse transcriptase, (Boehringer Mannheim, Indianapolis, IN)] was used to copy viral RNA into cDNA. The cDNA was then amplified using a thermostable DNA polymerase [e.g. Taq (Roche Molecular Systems, Inc., Branchburg, NJ), Tth (Roche Molecular

35 Systems, Inc., Branchburg, NJ), PrimeZyme (isolated from *Thermus brockianus*, Biometra, Gottingen, Germany)] or a

5 combination of thermostable polymerases as described for  
the performance of "long PCR" (Barnes, W.M., (1994) Proc.  
Natl. Acad. Sci, USA 91, 2216-2220) [e.g. Expand High  
Fidelity PCR System (Taq + Pwo), (Boehringer Mannheim.  
Indianapolis, IN) OR GeneAmp XL PCR kit (Tth + Vent),  
10 (Roche Molecular Systems, Inc., Branchburg, NJ)].

PCR6 (Table 5, #1) is used for reverse transcription of  
viral RNA into cDNA. The primers, ApaI primer (PDSApa,  
Table 5, #2) and AgeI primer (PDSAge, Table 5, #3) used to  
15 amplify the "test" patient-derived segments contained  
sequences resulting in ApaI and AgeI recognition sites  
being introduced into both ends of the PCR product,  
respectively.

Resistance test vectors incorporating the "test"  
20 patient-derived segments were constructed as described in  
US Patent Number 5,837,464 (International Publication  
Number WO 97/27319) (see Fig. 1) using an amplified DNA  
product of 1.5 kB prepared by RT-PCR using viral RNA as a  
template and oligonucleotides PCR6 (#1), PDSApa (#2) and  
25 PDSAge (#3) as primers, followed by digestion with ApaI  
and AgeI or the isoschizomer PinAI. To ensure that the  
plasmid DNA corresponding to the resultant resistance test  
vector comprises a representative sample of the HIV viral  
quasi-species present in the serum of a given patient,  
30 many (>100) independent E. coli transformants obtained in  
the construction of a given resistance test vector were  
pooled and used for the preparation of plasmid DNA.

A packaging expression vector encoding an amphotrophic  
35 MuLV 4070A env gene product enables production in a  
resistance test vector host cell of resistance test vector



5 viral particles which can efficiently infect human target  
cells. Resistance test vectors encoding all HIV genes  
with the exception of env were used to transfect a  
packaging host cell (once transfected the host cell is  
referred to as a resistance test vector host cell). The  
10 packaging expression vector which encodes the amphotrophic  
MuLV 4070A env gene product is used with the resistance  
test vector to enable production in the resistance test  
vector host cell of infectious pseudotyped resistance test  
vector viral particles.

15 Resistance tests performed with resistance test vectors  
were carried out using packaging host and target host  
cells consisting of the human embryonic kidney cell line  
293 (Cell Culture Facility, UC San Francisco, SF, CA) or  
20 the Jurkat leukemic T-cell line (Arthur Weiss, UC San  
Francisco, SF, CA).

Resistance tests were carried out with resistance test  
vectors using two host cell types. Resistance test vector  
25 viral particles were produced by a first host cell (the  
resistance test vector host cell) that was prepared by  
transfecting a packaging host cell with the resistance  
test vector and the packaging expression vector. The  
resistance test vector viral particles were then used to  
30 infect a second host cell (the target host cell) in which  
the expression of the indicator gene is measured (see Fig.  
2).

The resistance test vectors containing a functional  
35 luciferase gene cassette were constructed and host cells  
were transfected with the resistance test vector DNA. The



5 resistant test vectors contained patient-derived reverse  
transcriptase and protease DNA sequences that encode  
proteins which were either susceptible or resistant to the  
antiretroviral agents, such as nucleoside reverse  
transcriptase inhibitors, non-nucleoside reverse  
10 transcriptase inhibitors and protease inhibitors. The  
resistance test vector viral particles produced by  
transfecting the resistance test vector DNA into host  
cells, either in the presence or absence of protease  
inhibitors, were used to infect target host cells grown  
15 either in the absence of NRTI or NNRTI or in the presence  
of increasing concentrations of the drug. Luciferase  
activity in infected target host cells in the presence of  
drug was compared to the luciferase activity in infected  
target host cells in the absence of drug. Drug resistance  
20 was measured as the concentration of drug required to  
inhibit by 50% the luciferase activity detected in the  
absence of drug (inhibitory concentration 50%, IC<sub>50</sub> ).  
The IC<sub>50</sub> values were determined by plotting percent drug  
inhibition vs. log<sub>10</sub> drug concentration.

25 Host cells were seeded in 10-cm-diameter dishes and were  
transfected one day after plating with resistance test  
vector plasmid DNA and the envelope expression vector.  
Transfections were performed using a calcium-phosphate  
30 co-precipitation procedure. The cell culture media  
containing the DNA precipitate was replaced with fresh  
medium, from one to 24 hours, after transfection. Cell  
culture media containing resistance test vector viral  
particles was harvested one to four days after  
35 transfection and was passed through a 0.45-mm filter  
before being stored at -80°C. HIV capsid protein (p24)

5 levels in the harvested cell culture media were determined  
by an EIA method as described by the manufacturer (SIAC;  
Frederick, MD). Before infection, target cells (293 and  
293/T) were plated in cell culture media. Control  
10 infections were performed using cell culture media from  
mock transfections (no DNA) or transfections containing  
the resistance test vector plasmid DNA without the  
envelope expression plasmid. One to three or more days  
after infection the media was removed and cell lysis  
buffer (Promega) was added to each well. Cell lysates  
15 were assayed for luciferase activity. The inhibitory  
effect of the drug was determined using the following  
equation:

$$\% \text{ luciferase inhibition} = \frac{[1 - (\text{RLU}_{\text{luc}} [\text{drug}] / \text{RLU}_{\text{luc}})] \times 100}{20}$$

where  $\text{RLU}_{\text{luc}} [\text{drug}]$  is the relative light unit of  
luciferase activity in infected cells in the presence of  
drug and  $\text{RLU}_{\text{luc}}$  is the Relative Light Unit of luciferase  
25 activity in infected cells in the absence of drug. IC<sub>50</sub>  
values were obtained from the sigmoidal curves that were  
generated from the data by plotting the percent inhibition  
of luciferase activity vs. the  $\log_{10}$  drug concentration.  
Examples of drug inhibition curves are shown in (Fig. 3).

30

## EXAMPLE 2

**An in vitro Assay Using Resistance Test Vectors And Site  
Directed Mutants To Correlate Phenotypes And Genotypes  
Associated With HIV Drug Susceptibility And Resistance**

35 Phenotypic susceptibility analysis of patient HIV samples  
Resistance test vectors are constructed as described in

5 example 1. Resistance test vectors, or clones derived from  
the resistance test vector pools, are tested in a  
phenotypic assay to determine accurately and  
quantitatively the level of susceptibility to a panel of  
10 anti-retroviral drugs. This panel of anti-retroviral  
drugs may comprise members of the classes known as  
nucleoside-analog reverse transcriptase inhibitors  
(NRTIs), non-nucleoside reverse transcriptase inhibitors  
(NNRTIs), and protease inhibitors (PRIs). The panel of  
drugs can be expanded as new drugs or new drug targets  
15 become available. An IC50 is determined for each  
resistance test vector pool for each drug tested. The  
pattern of susceptibility to all of the drugs tested is  
examined and compared to known patterns of susceptibility.

20 A patient sample can be further examined for genotypic  
changes correlated with the pattern of susceptibility  
observed.

**Genotypic analysis of patient HIV samples**

Resistance test vector DNAs, either pools or clones, are  
25 analyzed by any of the genotyping methods described in  
Example 1. In one embodiment of the invention, patient  
HIV sample sequences are determined using viral RNA  
purification, RT/PCR and ABI chain terminator automated  
sequencing. The sequence that is determined is compared  
30 to control sequences present in the database or is  
compared to a sample from the patient prior to initiation  
of therapy, if available. The genotype is examined for  
sequences that are different from the control or  
pre-treatment sequence and correlated to the observed  
35 phenotype.

5     **Phenotypic susceptibility analysis of site directed mutants**

Genotypic changes that are observed to correlate with changes in phenotypic patterns of drug susceptibility are evaluated by construction of resistance test vectors containing the specific mutation on a defined, wild-type (drug susceptible) genetic background. Mutations may be incorporated alone and/or in combination with other mutations that are thought to modulate the susceptibility of HIV to a certain drug or class of drugs. Mutations are introduced into the resistance test vector through any of the widely known methods for site-directed mutagenesis. In one embodiment of this invention the mega-primer PCR method for site-directed mutagenesis is used. A resistance test vector containing the specific mutation or group of mutations are then tested using the phenotypic susceptibility assay described above and the susceptibility profile is compared to that of a genetically defined wild-type (drug susceptible) resistance test vector which lacks the specific mutations. Observed changes in the pattern of phenotypic susceptibility to the antiretroviral drugs tested are attributed to the specific mutations introduced into the resistance test vector.

30     **EXAMPLE 3**

**Using Resistance Test Vectors To Correlate Genotypes And Phenotypes Associated With Changes in PRI Drug Susceptibility in HIV.**

**Phenotypic analysis of Patient 0732**

35     A resistance test vector was constructed as described in example 1 from a patient sample designated as 0732. This

5 patient had been previously treated with nelfinavir.  
Isolation of viral RNA and RT/PCR was used to generate a  
patient derived segment that comprised viral sequences  
coding for all of PR and aa 1 - 313 of RT. The patient  
derived segment was inserted into an indicator gene viral  
10 vector to generate a resistance test vector designated  
RTV-0732. RTV-0732 was tested using a phenotypic  
susceptibility assay to determine accurately and  
quantitatively the level of susceptibility to a panel of  
anti-retroviral drugs. This panel of anti-retroviral  
15 drugs comprised members of the classes known as NRTIs  
(AZT, 3TC, d4T, ddI, ddC, and abacavir), NNRTIs  
(delavirdine, nevirapine and efavirenz), and PRIs  
(indinavir, nelfinavir, ritonavir, saquinavir and  
amprenavir). An IC50 was determined for each drug tested.  
20 Susceptibility of the patient virus to each drug was  
examined and compared to known patterns of susceptibility.  
A pattern of susceptibility to the PRIs was observed for  
patient sample RTV-0732 in which there was a decrease in  
both nelfinavir and indinavir susceptibility (increased  
25 resistance) and an increase in amprenavir susceptibility  
(see Fig. 4 and Table 1). Patient sample 0732 was examined  
further for genotypic changes associated with the pattern  
of susceptibility.

30 **Determination of genotype of patient 0732**

RTV-0732 DNA was analyzed by ABI chain terminator  
automated sequencing. The nucleotide sequence was  
compared to the consensus sequence of a wild type clade B  
HIV-1 (HIV Sequence Database Los Alamos, NM). The  
35 nucleotide sequence was examined for sequences that are  
different from the control sequence. PR mutations were

5 noted at positions K14R, I15V, K20T, E35D, M36I, R41K,  
I62V, L63Q and N88S. K14R, I15V, E35D, R41K and I62V are  
naturally occurring polymorphisms in HIV-1 PR and are not  
associated with reduced susceptibility to any drug. M36I  
has previously been described to be associated with  
10 resistance to ritonavir and nelfinavir (Shihazi, 1998).  
N88S has previously been described to be associated with  
resistance to nelfinavir (Patick AAC, 42: 2637 (1998) and  
an investigational PRI, SC55389A (Smidt, 1997)).

15 **Phenotypic analysis of Patient 627**

A resistance test vector was constructed as described in  
example 1 from a patient sample designated as 627. This  
patient had been treated with indinavir. Isolation of  
viral RNA and RT/PCR was used to generate a patient  
20 derived segment that comprised viral sequences coding for  
all of PR and aa 1 - 313 of RT. The patient derived  
segment was inserted into an indicator gene viral vector  
to generate a resistance test vector designated RTV-627.  
RTV-627 was tested using a phenotypic susceptibility assay  
25 to determine accurately and quantitatively the level of  
susceptibility to a panel of anti-retroviral drugs. This  
panel of anti-retroviral drugs comprised members of the  
classes known as NRTIs (AZT, 3TC, d4T, ddI, ddC, and  
abacavir), NNRTIs (delavirdine, nevirapine and efavirenz),  
30 and PRIs (indinavir, nelfinavir, ritonavir, saquinavir and  
amprenavir). An IC50 was determined for each drug tested.  
Susceptibility of the patient virus to each drug was  
examined and compared to known patterns of susceptibility.  
A pattern of susceptibility to the PRIs was observed for  
35 patient sample RTV-627 in which there was a decrease in  
indinavir and nelfinavir susceptibility (increased

5 resistance) and an increase in amprenavir and saquinavir susceptibility. Patient sample 627 was examined further for genotypic changes associated with the pattern of susceptibility.

10 **Determination of genotype of patient 627**

RTV-627 DNA was analyzed by ABI chain terminator automated sequencing. The nucleotide sequence was compared to the consensus sequence of a wild type clade B HIV-1 (HIV Sequence Database Los Alamos, NM). The nucleotide  
15 sequence was examined for sequences that are different from the control sequence. PR mutations were noted at positions 13I/V, E35D, M46L, L63P, I64V, I73V and N88S. I13V, E35D and I64V are naturally occurring polymorphisms in HIV-1 PR and are not associated with reduced  
20 susceptibility to any drug. M46L has previously been described to be associated with resistance to indinavir and amprenavir. L63P has previously been described to be associated with resistance to indinavir and nelfinavir. N88S has previously been described to be associated with  
25 resistance to nelfinavir (Patick, 1998) and an investigational PRI, SC55389A (Smidt, 1997).

**Phenotypic analysis of Patient 1208**

A resistance test vector was constructed as described in  
30 example 1 from a patient sample designated as 1208. This patient had been previously treated with nelfinavir. Isolation of viral RNA and RT/PCR was used to generate a patient derived segment that comprised viral sequences coding for all of PR and aa 1 - 313 of RT. The patient  
35 derived segment was inserted into an indicator gene viral vector to generate a resistance test vector designated



5 RTV-1208. RTV-1208 was tested using a phenotypic  
susceptibility assay to determine accurately and  
quantitatively the level of susceptibility to a panel of  
anti-retroviral drugs. This panel of anti-retroviral drugs  
10 comprised members of the classes known as NRTIs (AZT, 3TC,  
d4T, ddI, ddC, and abacavir), NNRTIs (delavirdine,  
nevirapine and efavirenz), and PRIs (indinavir,  
nelfinavir, ritonavir, saquinavir and amprenavir). An IC50  
was determined for each drug tested. Susceptibility of  
the patient virus to each drug was examined and compared  
15 to known patterns of susceptibility. A pattern of  
susceptibility to the PRIs was observed for patient sample  
RTV-1208 in which there was a decrease in indinavir and  
nelfinavir susceptibility (increased resistance) and an  
increase in amprenavir susceptibility. Patient sample  
20 1208 was examined further for genotypic changes associated  
with the pattern of susceptibility.

#### **Determination of genotype of patient 1208**

RTV-1208 DNA was analyzed by ABI chain terminator  
25 automated sequencing. The nucleotide sequence was  
compared to the consensus sequence of a wild type clade B  
HIV-1 (HIV Sequence Database Los Alamos, NM). The  
nucleotide sequence was examined for sequences that are  
different from the control sequence. PR mutations were  
30 noted at positions I62V, L63P, V77I, and N88S. I62V is a  
naturally occurring polymorphism in HIV-1 PR and is not  
associated with reduced susceptibility to any drug. L63P  
has previously been described to be associated with  
resistance to indinavir and nelfinavir. V77I has  
35 previously been described to be associated with resistance  
to nelfinavir. N88S has previously been described to be



5 associated with resistance to nelfinavir (Patick, 1998)  
and an investigational PRI, SC55389A (Smidt, 1997).

10 **Phenotypic analysis of Patient 360**

15 A resistance test vector was constructed as described in  
example 1 from a patient sample designated as 360. This  
patient had been previously treated with indinavir.  
Isolation of viral RNA and RT/PCR was used to generate a  
patient derived segment that comprised viral sequences  
coding for all of PR and aa 1 - 313 of RT. The patient  
derived segment was inserted into an indicator gene viral  
vector to generate a resistance test vector designated  
RTV-360. RTV-360 was tested using a phenotypic  
20 susceptibility assay to determine accurately and  
quantitatively the level of susceptibility to a panel of  
anti-retroviral drugs. This panel of anti-retroviral drugs  
comprised members of the classes known as NRTIs (AZT, 3TC,  
d4T, ddI, ddC, and abacavir), NNRTIs (delavirdine,  
25 nevirapine and efavirenz), and PRIs (indinavir,  
nelfinavir, ritonavir, saquinavir and amprenavir). An IC50  
was determined for each drug tested. Susceptibility of  
the patient virus to each drug was examined and compared  
to known patterns of susceptibility. A pattern of  
30 susceptibility to the PRIs was observed for patient sample  
RTV-360 in which there was a decrease in indinavir and  
nelfinavir susceptibility (increased resistance) and an  
increase in amprenavir susceptibility. Patient sample 360  
was examined further for genotypic changes associated with  
35 the pattern of susceptibility.

5      **Determination of genotype of patient 360**

RTV-360 DNA was analyzed by ABI chain terminator automated sequencing. The nucleotide sequence was compared to the consensus sequence of a wild type clade B HIV-1 (HIV Sequence Database Los Alamos, NM). The nucleotide  
10 sequence was examined for sequences that are different from the control sequence. PR mutations were noted at positions I13V, K20M, M36V, N37A, M46I, I62V, L63P, N88S, and I93L. I13V, N37A and I62V are naturally occurring polymorphisms in HIV-1 PR and are not associated with  
15 reduced susceptibility to any drug. K20M has previously been described to be associated with resistance to indinavir. M46I has previously been described to be associated with resistance to indinavir, ritonavir, nelfinavir and amprenavir. L63P has previously been  
20 described to be associated with resistance to indinavir and nelfinavir. N88S has previously been described to be associated with resistance to nelfinavir (Patick, 1998) and an investigational PRI, SC55389A (Smidt, 1997).

25      **Phenotypic analysis of Patient 0910**

A resistance test vector was constructed as described in example 1 from a patient sample designated as 0910. This patient had been previously treated with nelfinavir. Isolation of viral RNA and RT/PCR was used to generate a  
30 patient derived segment that comprised viral sequences coding for all of PR and aa 1 - 313 of RT. The patient derived segment was inserted into an indicator gene viral vector to generate a resistance test vector designated RTV-0910. RTV-0910 was tested using a phenotypic  
35 susceptibility assay to determine accurately and quantitatively the level of susceptibility to a panel of

5 anti-retroviral drugs. This panel of anti-retroviral drugs  
comprised members of the classes known as NRTIs (AZT, 3TC,  
d4T, ddI, ddC, and abacavir), NNRTIs (delavirdine,  
nevirapine and efavirenz), and PRIs (indinavir,  
nelfinavir, ritonavir, saquinavir and amprenavir). An IC50  
10 was determined for each drug tested. Susceptibility of  
the patient virus to each drug was examined and compared  
to known patterns of susceptibility. A pattern of  
susceptibility to the PRIs was observed for patient sample  
RTV-0910 in which there was a decrease in indinavir and  
15 nelfinavir susceptibility (increased resistance) and an  
increase in amprenavir susceptibility. Patient sample  
0910 was examined further for genotypic changes associated  
with the pattern of susceptibility.

20 **Determination of genotype of patient 0910**

RTV-0910 DNA was analyzed by ABI chain terminator  
automated sequencing. The nucleotide sequence was  
compared to the consensus sequence of a wild type clade B  
HIV-1 (HIV Sequence Database Los Alamos, NM). The  
25 nucleotide sequence was examined for sequences that are  
different from the control sequence. PR mutations were  
noted at positions M46I, L63P, V77I, N88S and I93I/L.  
I13V, K14R, N37D and I193L are naturally occurring  
polymorphism in HIV-1 PR and is not associated with  
30 reduced susceptibility to any drug. V77I has previously  
been described to be associated with resistance to  
nelfinavir. M46I has previously been described to be  
associated with resistance to indinavir, ritonavir,  
nelfinavir and amprenavir. L63P has previously been  
35 described to be associated with resistance to indinavir  
and nelfinavir. N88S has previously been described to be

5 associated with resistance to nelfinavir (Patick, 1998)  
and an investigational PRI, SC55389A (Smidt, 1997).

#### **Phenotypic analysis of Patient 3542**

10 A resistance test vector was constructed as described in  
example 1 from a patient sample designated as 3542. This  
patient had been treated with indinavir. Isolation of  
viral RNA and RT/PCR was used to generate a patient  
derived segment that comprised viral sequences coding for  
all of PR and aa 1 - 313 of RT. The patient derived  
15 segment was inserted into an indicator gene viral vector  
to generate a resistance test vector designated RTV-3542.  
RTV-3542 was tested using a phenotypic susceptibility  
assay to determine accurately and quantitatively the level  
of susceptibility to a panel of anti-retroviral drugs.  
20 This panel of anti-retroviral drugs comprised members of  
the classes known as NRTIs (AZT, 3TC, d4T, ddI, ddC, and  
abacavir), NNRTIs (delavirdine, nevirapine and efavirenz),  
and PRIs (indinavir, nelfinavir, ritonavir, saquinavir and  
amprenavir). An IC50 was determined for each drug tested.  
25 Susceptibility of the patient virus to each drug was  
examined and compared to known patterns of susceptibility.  
A pattern of susceptibility to the PRIs was observed for  
patient sample RTV-3542 in which there was a decrease in  
indinavir, nelfinavir and ritonavir susceptibility  
30 (increased resistance) and an increase in amprenavir  
susceptibility. Patient sample 3542 was examined further  
for genotypic changes associated with the pattern of  
susceptibility.

35 Determination of genotype of patient 3542  
RTV-3542 DNA was analyzed by ABI chain terminator

5 automated sequencing. The nucleotide sequence was  
compared to the consensus sequence of a wild type clade B  
HIV-1 (HIV Sequence Database Los Alamos, NM). The  
nucleotide sequence was examined for sequences that are  
different from the control sequence. PR mutations were  
10 noted at positions I13V, K14R, N37D, M46I, L63P, N88S and  
I93L. K14R and N37A/D are naturally occurring  
polymorphisms in HIV-1 PR and are not associated with  
reduced susceptibility to any drug. M46I has previously  
been described to be associated with resistance to  
15 indinavir, ritonavir, nelfinavir and amprenavir. L63P has  
previously been described to be associated with resistance  
to indinavir and nelfinavir. N88S has previously been  
described to be associated with resistance to nelfinavir  
(Patick, 1998) and an investigational PRI, SC55389A  
20 (Smidt, 1997).

#### **Phenotypic analysis of Patient 3654**

A resistance test vector was constructed as described in  
example 1 from a patient sample designated as 3654. This  
25 patient had been previously treated with ritonavir.  
Isolation of viral RNA and RT/PCR was used to generate a  
patient derived segment that comprised viral sequences  
coding for all of PR and aa 1 - 313 of RT. The patient  
derived segment was inserted into an indicator gene viral  
30 vector to generate a resistance test vector designated  
RTV-3654. RTV-3654 was tested using a phenotypic  
susceptibility assay to determine accurately and  
quantitatively the level of susceptibility to a panel of  
anti-retroviral drugs. This panel of anti-retroviral drugs  
35 comprised members of the classes known as NRTIs (AZT, 3TC,  
d4T, ddI, ddC, and abacavir), NNRTIs (delavirdine,

5 nevirapine and efavirenz), and PRIs (indinavir,  
nelfinavir, ritonavir, saquinavir and amprenavir). An IC50  
was determined for each drug tested. Susceptibility of  
the patient virus to each drug was examined and compared  
to known patterns of susceptibility. A pattern of  
10 susceptibility to the PRIs was observed for patient sample  
RTV-3654 in which there was a decrease in indinavir and  
nelfinavir susceptibility (increased resistance) and an  
increase in amprenavir susceptibility. Patient sample  
3654 was examined further for genotypic changes associated  
15 with the pattern of susceptibility.

**Determination of genotype of patient 3654**

RTV-3654 DNA was analyzed by ABI chain terminator  
automated sequencing. The nucleotide sequence was  
20 compared to the consensus sequence of a wild type clade B  
HIV-1 (HIV Sequence Database Los Alamos, NM). The  
nucleotide sequence was examined for sequences that are  
different from the control sequence. PR mutations were  
noted at positions I13V, R41K, M46I, L63P, V77I, N88S and  
25 I93L. I13V, R41K and I93L are naturally occurring  
polymorphism in HIV-1 PR and is not associated with  
reduced susceptibility to any drug. M46I has previously  
been described to be associated with resistance to  
indinavir, ritonavir, nelfinavir and amprenavir. L63P has  
30 previously been described to be associated with resistance  
to indinavir and nelfinavir. V77I has previously been  
described to be associated with resistance to nelfinavir.  
N88S has previously been described to be associated with  
resistance to an investigational PRI, SC55389A (Smidt,  
35 1997).

5

**EXAMPLE 4**

**Using Site Directed Mutants To Correlate Genotypes And  
Phenotypes Associated With Changes in PRI Drug  
Susceptibility in HIV.**

**Site directed mutagenesis**

10 Resistance test vectors were constructed containing the  
N88S mutation alone and in combination with other  
substitutions in PR (L63P, V77I and M46L) known to  
modulate the HIV susceptibility to PRIs. Mutations were  
introduced into the resistance test vector using the  
15 mega-primer PCR method for site-directed mutagenesis.  
(Sakar G and Sommar SS (1994) Biotechniques 8(4),  
404-407). First, a resistance test vector was constructed  
that harbors a unique RsrII restriction site 590 bp  
downstream of the ApaI restriction site. The 590 bp ApaI  
20 - RsrII fragment thus contains the entire protease region.  
This site was introduced by site-specific  
oligonucleotide-directed mutagenesis using primer #4. All  
subsequent mutants were constructed by fragment-exchange  
of the wild-type ApaI - RsrII fragment in the parent  
25 vector with the equivalent fragment carrying the  
respective mutations.

A resistance test vector containing the N88S mutation  
(N88S-RTV) was tested using the phenotypic susceptibility  
30 assay described above and the results were compared to  
that of a genetically defined resistance test vector that  
was wild type at position 88. The pattern of phenotypic  
susceptibility to the PRIs in the N88S-RTV was altered as  
compared to wild type. In the context of an otherwise  
35 wild type background (i.e. N88S mutation alone) the  
N88S-RTV was more susceptible to both amprenavir and



5     ritonavir and slightly less susceptible to nelfinavir  
compared to the wild type control RTV (see Table 2).

10     A resistance test vector containing the N88S mutation  
along with the L63P mutation (L63P-N88S-RTV) was tested  
using the phenotypic susceptibility assay described above  
and the results were compared to that of a genetically  
defined resistance test vector that was wild type at  
positions 63 and 88. The L63P-N88S-RTV showed decreased  
susceptibility to both indinavir and nelfinavir and an  
15     increase in the susceptibility to amprenavir compared the  
wild-type control RTV (see Table 2). Thus it appears that  
the introduction of a second mutation, L63P, in addition  
to N88S, results in a reduction in susceptibility to  
nelfinavir and indinavir while the increased  
20     susceptibility to amprenavir is maintained.

25     A resistance test vector containing the N88S mutation  
along with the L63P mutation and the V77I mutation  
(L63P-V77I-N88S-RTV) was tested using the phenotypic  
susceptibility assay described above and the results were  
compared to that of a genetically defined resistance test  
vector that was wild type at positions 63 and 77 and 88.  
The RTV containing mutations at these positions,  
L63P-V77I-N88S-RTV, showed a decrease in susceptibility to  
30     both indinavir and nelfinavir and an increase in the  
susceptibility to amprenavir compared to the wild-type  
control RTV (see Fig. 5 and Table 2). Thus it appears  
that the introduction of a third mutation, V77I, in  
addition to L63P and N88S, results in a reduction in  
35     susceptibility to nelfinavir and indinavir while the  
increased susceptibility to amprenavir is maintained.



5

The N88S mutation was also introduced into an RTV containing additional mutations at positions L63P and M46L (M46L + L63P + N88S). The RTV containing mutations at these positions, M46L-L63P-N88S-RTV showed a decrease in susceptibility to nelfinavir and a slight decrease in susceptibility to indinavir and an increase in the susceptibility to amprenavir compared to the wild-type control RTV (see Fig. 5 and Table 2). Thus it appears that the introduction of a third mutation, M46L, in addition to L63P and N88S, results in a reduction in susceptibility to nelfinavir and indinavir while the increased susceptibility to amprenavir is maintained.

A resistance test vector containing the N88S mutation along with the M46L mutation, the L63P mutation, and the V77I mutation (M46L-L63P-V77I-N88S-RTV) was tested using the phenotypic susceptibility assay described above and the results were compared to that of a genetically defined resistance test vector that was wild type at positions 46, 63, 77 and 88. The RTV containing mutations at these four positions, M46L-L63P-V77I-N88S-RTV showed a decrease in susceptibility to nelfinavir and indinavir and an increase in the susceptibility to amprenavir compared to the wild-type control RTV (see Fig. 5 and Table 2). Thus it appears that the introduction of a fourth mutation, V77I, in addition to L63P, M46L and N88S results in a reduction in susceptibility to nelfinavir and indinavir while the increased susceptibility to amprenavir is maintained.

A resistance test vector containing the L63P mutation (L63P-RTV) was tested using the phenotypic susceptibility assay described above and the results were compared to

5 that of a genetically defined resistance test vector that was wild type at position 63. The pattern of phenotypic susceptibility to the PRIs in the L63P-RTV was similar to wild type with no significant changes in susceptibility to the PRIs observed.

10

The L63P mutation was also introduced into an RTV containing an additional mutation at position V77I. The L63P-V77I-RTV showed a slight decrease in susceptibility to nelfinavir compared to the wild-type control RTV (see Fig. 5 and Table 2).

15

#### EXAMPLE 5

##### **Predicting Response to Protease Inhibitors by Characterization of Amino Acid 88 of HIV-1 Protease.**

20

In one embodiment of this invention, changes in the amino acid at position 88 of the protease protein of HIV-1 is evaluated using the following method comprising: (i) collecting a biological sample from an HIV-1 infected subject; (ii) evaluating whether the biological sample contains nucleic acid encoding HIV-1 protease having an asparagine to serine mutation at codon 88 (N88S); and (iii) determining susceptibility to protease inhibitors (PRI).

25

30

The biological sample comprises whole blood, blood components including peripheral mononuclear cells (PBMC), serum, plasma (prepared using various anticoagulants such as EDTA, acid citrate-dextrose, heparin), tissue biopsies, cerebral spinal fluid (CSF), or other cell, tissue or body fluids. In another embodiment, the HIV-1 nucleic acid

35

5 (genomic RNA) or reverse transcriptase protein can be  
isolated directly from the biological sample or after  
purification of virus particles from the biological  
sample. Evaluating whether the amino acid at position 88  
of the HIV-1 protease is mutated to serine, can be  
10 performed using various methods, such as direct  
characterization of the viral nucleic acid encoding  
protease or direct characterization of the protease  
protein itself. Defining the amino acid at position 88 of  
protease can be performed by direct characterization of  
15 the protease protein by conventional or novel amino acid  
sequencing methodologies, epitope recognition by  
antibodies or other specific binding proteins or  
compounds. Alternatively, the amino acid at position 88  
of the HIV-1 protease protein can be defined by  
20 characterizing amplified copies of HIV-1 nucleic acid  
encoding the protease protein. Amplification of the HIV-1  
nucleic acid can be performed using a variety of  
methodologies including reverse transcription-polymerase  
chain reaction (RT-PCR), NASBA, SDA, RCR, or 3SR. The  
25 nucleic acid sequence encoding HIV protease at codon 88  
can be determined by direct nucleic acid sequencing using  
various primer extension-chain termination (Sanger, ABI/PE  
and Visible Genetics) or chain cleavage (Maxam and  
Gilbert) methodologies or more recently developed  
30 sequencing methods such as matrix assisted laser  
desorption-ionization time of flight (MALDI-TOF) or mass  
spectrometry (Sequenom, Gene Trace Systems).  
Alternatively, the nucleic acid sequence encoding amino  
acid position 88 can be evaluated using a variety of probe  
35 hybridization methodologies, such as genechip  
hybridization sequencing (Affymetrix), line probe assay

5 (LiPA; Murex), and differential hybridization (Chiron).

10 In a preferred embodiment of this invention, evaluation of protease inhibitor susceptibility and of whether amino acid position 88 of HIV-1 protease was wild type or serine was carried out using a phenotypic susceptibility assay or genotypic assay, respectively, using resistance test vector DNA prepared from the biological sample. In one embodiment, the plasma sample was collected, viral RNA was purified and an RT-PCR methodology was used to amplify a patient derived segment encoding the HIV-1 protease and reverse transcriptase regions. The amplified patient derived segments were then incorporated, via DNA ligation and bacterial transformation, into an indicator gene viral vector thereby generating a resistance test vector.

20 Resistance test vector DNA was isolated from the bacterial culture and the phenotypic susceptibility assay was carried out as described in Example 1. The results of the phenotypic susceptibility assay with a patient sample having an N88S mutation in PR is shown in Figure 4. The

25 nucleic acid (DNA) sequence of the patient derived HIV-1 protease and reverse transcriptase regions from patient sample 0732 was determined using a fluorescence detection chain termination cycle sequencing methodology (ABI/PE). The method was used to determine a consensus nucleic acid

30 sequence representing the combination of sequences of the mixture of HIV-1 variants existing in the subject sample (representing the quasispecies), and to determine the nucleic acid sequences of individual variants.

35 **Phenotypic and genotypic correlation of mutations at amino acid 88 of HIV-1 Protease.**

5 Phenotypic susceptibility profiles of patient samples and  
site directed mutants showed that amprenavir  
susceptibility correlated with the presence of the N88S  
mutation in HIV-1 protease. Phenotypic susceptibility  
profiles of patient samples and site directed mutants  
10 showed that a significant increase in amprenavir  
susceptibility (decreased resistance) correlated with a  
mutation in the nucleic acid sequence encoding the amino  
acid serine (S) at position 88 of HIV-1 protease.

15 Phenotypic susceptibility profiles of patient samples and  
site directed mutants showed reduction in amprenavir  
susceptibility (decreased resistance) and a decrease in  
susceptibility to nelfinavir and indinavir with the amino  
acid serine at position 88 when the PR mutations at  
20 positions 63, 77 or 46 were also present (L63P, V77I, or  
M46L).

#### EXAMPLE 6

25 **Using Resistance test vectors and site directed mutants to  
correlate genotypes associated with alterations in PRI  
susceptibility with viral fitness.**

Luciferase activity measured in the absence of drug for  
30 the seven resistance test vectors constructed from the  
patient viruses containing the N88S PR mutation ranged  
from 0.7 to 16% of control (Table 3). Although these  
viruses also contain multiple mutations in reverse  
transcriptase, which could also contribute to a reduction  
35 in viral fitness, the data suggest that viruses containing  
the N88S mutation are less fit than wild type. To confirm

5 this observation, the luciferase expression level for the site-directed mutant resistance test vectors was also examined.

10 Viruses containing N88S as the only substitution produced only 1.0% of the luciferase activity in the absence of drug (Table 4). This reduction was substantially alleviated by the addition of the L63P substitution (20.7%) or by addition of the combinations of L63P/V77I (29.3%) or M46L/L63P (28.0%). The L63P or L63P/V77I  
15 mutants had equivalent or increased relative luciferase activity compared to wild type (163.9 and 75.6%, respectively).

20 When the K20T substitution was added to the N88S background, either alone or in combination with L63P, only background levels of luciferase activity was detected. Sequence analysis confirmed the absence of additional mutations, which might render the vector inactive. Thus the combination of the K20T and N88S substitutions  
25 correlates with a severe defect in fitness.

#### EXAMPLE 7

##### Predicting Response to Protease Inhibitors by Characterization of Amino Acid 82 of HIV-1 30 Protease.

In one embodiment of this invention, changes in the amino acid at position 82 of the protease protein of HIV-1 are evaluated using the following method comprising: (i)  
35 collecting a biological sample from an HIV-1 infected subject; (ii) evaluating whether the biological sample

5 contains nucleic acid encoding HIV-1 protease having a  
valine to alanine (V82A), phenylalanine (V82F), serine  
(V82S), or threonine (V82T) substitution at codon 82; and  
(iii) determining susceptibility to protease inhibitors  
(PRI).

10

The biological sample comprises whole blood, blood  
components including peripheral mononuclear cells (PBMC),  
serum, plasma (prepared using various anticoagulants such  
as EDTA, acid citrate-dextrose, heparin), tissue biopsies,  
15 cerebral spinal fluid (CSF), or other cell, tissue or body  
fluids. In another embodiment, the HIV-1 nucleic acid  
(genomic RNA) or reverse transcriptase protein can be  
isolated directly from the biological sample or after  
purification of virus particles from the biological  
20 sample. Evaluating whether the amino acid at position 82  
of the HIV-1 protease is mutated to alanine,  
phenylalanine, serine, or threonine, can be performed  
using various methods, such as direct characterization of  
the viral nucleic acid encoding protease or direct  
25 characterization of the protease protein itself. Defining  
the amino acid at position 82 of protease can be performed  
by direct characterization of the protease protein by  
conventional or novel amino acid sequencing methodologies,  
epitope recognition by antibodies or other specific  
30 binding proteins or compounds. Alternatively, the amino  
acid at position 82 of the HIV-1 protease protein can be  
defined by characterizing amplified copies of HIV-1  
nucleic acid encoding the protease protein. Amplification  
of the HIV-1 nucleic acid can be performed using a variety  
35 of methodologies including reverse  
transcription-polymerase chain reaction (RT-PCR), NASBA,



5 SDA, RCR, or 3SR. The nucleic acid sequence encoding HIV  
protease at codon 82 can be determined by direct nucleic  
acid sequencing using various primer extension-chain  
termination (Sanger, ABI/PE and Visible Genetics) or chain  
10 cleavage (Maxam and Gilbert) methodologies or more  
recently developed sequencing methods such as matrix  
assisted laser desorption-ionization time of flight  
(MALDI-TOF) or mass spectrometry (Sequenom, Gene Trace  
Systems). Alternatively, the nucleic acid sequence  
15 encoding amino acid position 82 can be evaluated using a  
variety of probe hybridization methodologies, such as  
genechip hybridization sequencing (Affymetrix), line probe  
assay (LiPA; Murex), and differential hybridization  
(Chiron).

20 In a preferred embodiment of this invention, evaluation of  
protease inhibitor susceptibility and of whether amino  
acid position 82 of HIV-1 protease was wild type or  
alanine, phenylalanine, serine, or threonine, was carried  
out using a phenotypic susceptibility assay or genotypic  
25 assay, respectively, using resistance test vector DNA  
prepared from the biological sample. In one embodiment,  
the plasma sample was collected, viral RNA was purified  
and an RT-PCR methodology was used to amplify a patient  
derived segment encoding the HIV-1 protease and reverse  
30 transcriptase regions. The amplified patient derived  
segments were then incorporated, via DNA ligation and  
bacterial transformation, into an indicator gene viral  
vector thereby generating a resistance test vector.  
Resistance test vector DNA was isolated from the bacterial  
35 culture and the phenotypic susceptibility assay was  
carried out and analyzed as described in Example 1.



5

The nucleic acid (DNA) sequence of the patient derived HIV-1 protease and reverse transcriptase regions was determined using a fluorescence detection chain termination cycle sequencing methodology (ABI/PE). The method was used to determine a consensus nucleic acid sequence representing the combination of sequences of the mixture of HIV-1 variants existing in the subject sample (representing the quasispecies), and to determine the nucleic acid sequences of individual variants. Genotypes are analyzed as lists of amino acid differences between virus in the patient sample and a reference laboratory strain of HIV-1, NL4-3. Genotypes and corresponding phenotypes (fold-change in IC50 values) are entered in a relational database linking these two results with patient information. Large datasets can then be assembled from patient virus samples sharing particular characteristics, such as the presence of any given mutation, or combination of mutations or reduced susceptibility to any drug or combination of drugs.

25

**(a) Protease inhibitor susceptibility of viruses containing mutations at amino acid 82 of HIV-1 Protease.**

Phenotypic susceptibility profiles of 75 patient virus samples which contained a mutation at position 82 (V82A, F, S, or T), but no other primary mutations, were analyzed. According to most published guidelines, such viruses are expected to be resistant to ritonavir, nelfinavir, indinavir, and saquinavir. However, 8%, 20%, 23%, and 73% of these samples were phenotypically susceptible to these four protease inhibitors,

5        respectively (see Table 6).        Thus, particularly for  
indinavir and saquinavir, there was poor correlation  
between the presence of mutations at position 82 and drug  
susceptibility.

10        **(b) Indinavir susceptibility of viruses containing  
combinations of mutations at amino acid 82 and one  
secondary mutation in HIV-1 Protease.**

Indinavir resistance in viruses containing mutations at  
position 82 was evaluated with respect to the presence of  
15        other specific mutations.        Decreased indinavir  
susceptibility (fold-change in  $IC_{50}$  greater than 2.5) in  
viruses containing V82A, F, S, or T but no other primary  
mutations was correlated with the presence of mutations at  
secondary positions. Reduced indinavir susceptibility was  
20        observed in 20 samples containing mutations at both  
positions 24 and 82 (100%) and in 27 samples with both 71  
and 82 (100%) (See Table 7). The combination of mutations  
at position 82 with mutations at other positions (e.g. 54,  
46, 10, and 63) also significantly increased the  
25        proportion of samples that had reduced indinavir  
susceptibility (Table 7).

30        **(c) Saquinavir susceptibility of viruses containing  
combinations of mutations at amino acid 82 and one  
secondary mutation in HIV-1 Protease.**

Saquinavir resistance in viruses containing mutations at  
position 82 was evaluated with respect to the presence of  
other specific mutations.        Decreased saquinavir  
susceptibility (fold-change in  $IC_{50}$  greater than 2.5) in  
35        viruses containing V82A, F, S, or T but no other primary  
mutations was correlated with the presence of mutations at

5 secondary positions. Reduced saquinavir susceptibility was  
observed in 4 of 5 samples containing mutations at both  
positions 20 and 82 (80%) and in 8 of 11 samples with both  
36 and 82 (73%) (See Table 8). The combination of  
10 mutations at position 82 with mutations at other positions  
(e.g. 24, 71, 54, and 10) also significantly increased the  
proportion of samples that had reduced saquinavir  
susceptibility (Table 8).

15 **(d) Indinavir susceptibility of viruses containing  
combinations of mutations at amino acid 82 and many  
secondary mutations in HIV-1 Protease.**

Indinavir resistance in viruses containing mutations at  
position 82 was evaluated with respect to the presence of  
a defined number of other mutations. Decreased indinavir  
20 susceptibility (fold-change in  $IC_{50}$  greater than 2.5) in  
viruses containing V82A, F, S, or T but no other primary  
mutations was correlated with the number of mutations at  
secondary positions. Reduced indinavir susceptibility was  
observed in 100% of samples with V82A, F, S, or T and at  
25 least 6 other secondary mutations (See Table 9). The  
proportion of samples that had reduced indinavir  
susceptibility increased significantly in samples with  
V82A, F, S, or T combined with 3 to 5 other secondary  
mutations (Table 9).

30

**(e) Saquinavir susceptibility of viruses containing  
combinations of mutations at amino acid 82 and many  
secondary mutations in HIV-1 Protease.**

35 Saquinavir resistance in viruses containing mutations at  
position 82 was evaluated with respect to the presence of  
a defined number of other mutations. Decreased saquinavir

5 susceptibility (fold-change in  $IC_{50}$  greater than 2.5) in  
viruses containing V82A, F, S, or T but no other primary  
mutations was correlated with the number of mutations at  
secondary positions. Reduced saquinavir susceptibility  
was observed in 60 to 76% of samples with V82A, F, S, or T  
10 and at least 5 other secondary mutations (See Table 9).  
The proportion of samples that had reduced saquinivir  
susceptibility increased significantly in samples with  
V82A, F, S, or T combined with 3 or 4 other secondary  
mutations (Table 9).

15 **EXAMPLE 8**

**Predicting Response to Protease Inhibitors by  
Characterization of Amino Acid 90 of HIV-1  
Protease.**

20 In one embodiment of this invention, changes in the amino  
acid at position 90 of the protease protein of HIV-1 are  
evaluated using the following method comprising: (i)  
collecting a biological sample from an HIV-1 infected  
25 subject; (ii) evaluating whether the biological sample  
contains nucleic acid encoding HIV-1 protease having a  
leucine to methionine (L90M) substitution at codon 90; and  
(iii) determining susceptibility to protease inhibitors  
(PRI).

30 The biological sample comprises whole blood, blood  
components including peripheral mononuclear cells (PBMC),  
serum, plasma (prepared using various anticoagulants such  
as EDTA, acid citrate-dextrose, heparin), tissue biopsies,  
35 cerebral spinal fluid (CSF), or other cell, tissue or body  
fluids. In another embodiment, the HIV-1 nucleic acid

5 (genomic RNA) or reverse transcriptase protein can be  
isolated directly from the biological sample or after  
purification of virus particles from the biological  
sample. Evaluating whether the amino acid at position 90  
of the HIV-1 protease is mutated to methionine, can be  
10 performed using various methods, such as direct  
characterization of the viral nucleic acid encoding  
protease or direct characterization of the protease  
protein itself. Defining the amino acid at position 90 of  
protease can be performed by direct characterization of  
15 the protease protein by conventional or novel amino acid  
sequencing methodologies, epitope recognition by  
antibodies or other specific binding proteins or  
compounds. Alternatively, the amino acid at position 90  
of the HIV-1 protease protein can be defined by  
20 characterizing amplified copies of HIV-1 nucleic acid  
encoding the protease protein. Amplification of the HIV-1  
nucleic acid can be performed using a variety of  
methodologies including reverse transcription-polymerase  
chain reaction (RT-PCR), NASBA, SDA, RCR, or 3SR. The  
25 nucleic acid sequence encoding HIV protease at codon 90  
can be determined by direct nucleic acid sequencing using  
various primer extension-chain termination (Sanger, ABI/PE  
and Visible Genetics) or chain cleavage (Maxam and  
Gilbert) methodologies or more recently developed  
30 sequencing methods such as matrix assisted laser  
desorption-ionization time of flight (MALDI-TOF) or mass  
spectrometry (Sequenom, Gene Trace Systems).  
Alternatively, the nucleic acid sequence encoding amino  
acid position 90 can be evaluated using a variety of probe  
35 hybridization methodologies, such as genechip  
hybridization sequencing (Affymetrix), line probe assay

5 (LiPA; Murex), and differential hybridization (Chiron).

10 In a preferred embodiment of this invention, evaluation of protease inhibitor susceptibility and of whether amino acid position 90 of HIV-1 protease was wild type or methionine, was carried out using a phenotypic susceptibility assay or genotypic assay, respectively, using resistance test vector DNA prepared from the biological sample. In one embodiment, the plasma sample was collected, viral RNA was purified and an RT-PCR methodology was used to amplify a patient derived segment encoding the HIV-1 protease and reverse transcriptase regions. The amplified patient derived segments were then incorporated, via DNA ligation and bacterial transformation, into an indicator gene viral vector thereby generating a resistance test vector. Resistance test vector DNA was isolated from the bacterial culture and the phenotypic susceptibility assay was carried out and analyzed as described in Example 1.

25 The nucleic acid (DNA) sequence of the patient derived HIV-1 protease and reverse transcriptase regions was determined using a fluorescence detection chain termination cycle sequencing methodology (ABI/PE). The method was used to determine a consensus nucleic acid sequence representing the combination of sequences of the mixture of HIV-1 variants existing in the subject sample (representing the quasispecies), and to determine the nucleic acid sequences of individual variants. Genotypes are analyzed as lists of amino acid differences between virus in the patient sample and a reference laboratory strain of HIV-1, NL4-3. Genotypes and corresponding

35

5 phenotypes (fold-change in IC<sub>50</sub> values) are entered in a  
relational database linking these two results with patient  
information. Large datasets can then be assembled from  
patient virus samples sharing particular characteristics,  
such as the presence of any given mutation, or combination  
10 of mutants, or reduced susceptibility to any drug or  
combination of drugs.

**(a) Protease inhibitor susceptibility of viruses  
containing mutations at amino acid 90 of HIV-1 Protease.**

15 Phenotypic susceptibility profiles of 58 patient virus  
samples which contained a mutation at position 90 (L90M),  
but no other primary mutations, were analyzed. According  
to most published guidelines, such viruses are expected to  
20 be resistant to ritonavir, nelfinavir, indinavir, and  
saquinavir. However, 28%, 9%, 31%, and 47% of these  
samples were phenotypically susceptible to these four  
protease inhibitors, respectively (see Table 6). Thus,  
particularly for indinavir and saquinavir, there was poor  
25 correlation between the presence of mutations at position  
90 and drug susceptibility.

**(b) Indinavir susceptibility of viruses containing  
combinations of mutations at amino acid 90 and one  
30 secondary mutation in HIV-1 Protease.**

Indinavir resistance in viruses containing mutations at  
position 90 was evaluated with respect to the presence of  
other specific mutations. Decreased indinavir  
susceptibility (fold-change in IC<sub>50</sub> greater than 2.5) in  
35 viruses containing L90M but no other primary mutations was  
correlated with the presence of mutations at secondary



5 positions. Reduced indinavir susceptibility was observed  
in 17 of 19 samples containing mutations at both positions  
73 and 90 (89%) and in 16 of 18 samples with both 71 and  
90 (89%) (See Table 10). The combination of mutations at  
10 position 90 with mutation at position 46 also  
significantly increased the proportion of samples that had  
reduced indinavir susceptibility (Table 10).

15 **(c) Saquinavir susceptibility of viruses containing  
combinations of mutations at amino acid 90 and one  
secondary mutation in HIV-1 Protease.**

Saquinavir resistance in viruses containing mutations at  
position 90 was evaluated with respect to the presence of  
other specific mutations. Decreased saquinavir  
susceptibility (fold-change in  $IC_{50}$  greater than 2.5) in  
20 viruses containing L90M but no other primary mutations was  
correlated with the presence of mutations at secondary  
positions. Reduced saquinavir susceptibility was observed  
in 15 of 19 samples containing mutations at both positions  
73 and 90 (79%) and in 14 of 18 samples with both 71 and  
25 90 (78%) (See Table 11). The combination of mutations at  
position 90 with mutations at other positions (e.g. 77 and  
10) also significantly increased the proportion of samples  
that had reduced saquinavir susceptibility (Table 1).

30

35 **(d) Indinavir susceptibility of viruses containing  
combinations of mutations at amino acid 90 and many  
secondary mutations in HIV-1 Protease.**

Indinavir resistance in viruses containing mutations at

5 position 90 was evaluated with respect to the presence of  
a defined number of other mutations. Decreased indinavir  
susceptibility (fold-change in  $IC_{50}$  greater than 2.5) in  
viruses containing L90M but no other primary mutations was  
correlated with the number of mutations at secondary  
10 positions. Reduced indinavir susceptibility was observed  
in 100% of samples with L90M and at least 5 other  
secondary mutations had (See Table 12). The proportion of  
samples that had reduced indinavir susceptibility  
increased significantly in samples with L90M combined with  
15 3 or 4 other secondary mutations (Table 12).

**(e) Saquinavir susceptibility of viruses containing combinations of mutations at amino acid 90 and many secondary mutations in HIV-1 Protease.**

20 Saquinavir resistance in viruses containing mutations at  
position 90 was evaluated with respect to the presence of  
a defined number of other mutations. Decreased saquinavir  
susceptibility (fold-change in  $IC_{50}$  greater than 2.5) in  
viruses containing L90M but no other primary mutations was  
25 correlated with the number of mutations at secondary  
positions. Reduced saquinavir susceptibility was observed  
in 100% of samples with L90M and at least 5 other  
secondary mutations (See Table 12). The proportion of  
samples that had reduced saquinivir susceptibility  
30 increased significantly in samples with L90M combined with  
3 or 4 other secondary mutations (Table 12).

**EXAMPLE 9**

**Predicting Response to Protease Inhibitors by  
35 Characterization of Amino Acids 82 and 90 of HIV-1  
Protease.**

5

In one embodiment of this invention, changes in the amino acid at position 82 and 90 of the protease protein of HIV-1 are evaluated using the following method comprising:  
(i) collecting a biological sample from an HIV-1 infected  
10 subject; (ii) evaluating whether the biological sample contains nucleic acid encoding HIV-1 protease having a valine to alanine (V82A), phenylalanine (V82F), serine (V82S), or threonine (V82T) substitution at codon 82 or a leucine to methionine at position 90 (L90M); and (iii)  
15 determining susceptibility to protease inhibitors (PRI).

The biological sample comprises whole blood, blood components including peripheral mononuclear cells (PBMC), serum, plasma (prepared using various anticoagulants such  
20 as EDTA, acid citrate-dextrose, heparin), tissue biopsies, cerebral spinal fluid (CSF), or other cell, tissue or body fluids. In another embodiment, the HIV-1 nucleic acid (genomic RNA) or reverse transcriptase protein can be isolated directly from the biological sample or after  
25 purification of virus particles from the biological sample. Evaluating whether the amino acid at position 82 of the HIV-1 protease is mutated to alanine, phenylalanine, serine, or threonine or at position 90 to methionine, can be performed using various methods, such  
30 as direct characterization of the viral nucleic acid encoding protease or direct characterization of the protease protein itself. Defining the amino acid at positions 82 and 90 of protease can be performed by direct characterization of the protease protein by conventional  
35 or novel amino acid sequencing methodologies, epitope recognition by antibodies or other specific binding

5 proteins or compounds. Alternatively, the amino acid at  
positions 82 and 90 of the HIV-1 protease protein can be  
defined by characterizing amplified copies of HIV-1  
nucleic acid encoding the protease protein. Amplification  
10 of methodologies including reverse  
transcription-polymerase chain reaction (RT-PCR), NASBA,  
SDA, RCR, or 3SR. The nucleic acid sequence encoding HIV  
protease at codons 82 and 90 can be determined by direct  
nucleic acid sequencing using various primer  
15 extension-chain termination (Sanger, ABI/PE and Visible  
Genetics) or chain cleavage (Maxam and Gilbert)  
methodologies or more recently developed sequencing  
methods such as matrix assisted laser  
desorption-ionization time of flight (MALDI-TOF) or mass  
20 spectrometry (Sequenom, Gene Trace Systems).  
Alternatively, the nucleic acid sequence encoding amino  
acid positions 82 and 90 can be evaluated using a variety  
of probe hybridization methodologies, such as genechip  
hybridization sequencing (Affymetrix), line probe assay  
25 (LiPA; Murex), and differential hybridization (Chiron).

In a preferred embodiment of this invention, evaluation of  
protease inhibitor susceptibility and of whether amino  
acid positions 82 and 90 of HIV-1 protease was wild type  
30 or alanine, phenylalanine, serine, or threonine in the  
case of position 82 and methionine at position 90, was  
carried out using a phenotypic susceptibility assay or  
genotypic assay, respectively, using resistance test  
vector DNA prepared from the biological sample. In one  
35 embodiment, plasma sample was collected, viral RNA was  
purified and an RT-PCR methodology was used to amplify a

5 patient derived segment encoding the HIV-1 protease and  
reverse transcriptase regions. The amplified patient  
derived segments were then incorporated, via DNA ligation  
and bacterial transformation, into an indicator gene viral  
vector thereby generating a resistance test vector.  
10 Resistance test vector DNA was isolated from the bacterial  
culture and the phenotypic susceptibility assay was  
carried out and analyzed as described in Example 1.

The nucleic acid (DNA) sequence of the patient derived  
15 HIV-1 protease and reverse transcriptase regions was  
determined using a fluorescence detection chain  
termination cycle sequencing methodology (ABI/PE). The  
method was used to determine a consensus nucleic acid  
sequence representing the combination of sequences of the  
20 mixture of HIV-1 variants existing in the subject sample  
(representing the quasispecies), and to determine the  
nucleic acid sequences of individual variants. Genotypes  
are analyzed as lists of amino acid differences between  
virus in the patient sample and a reference laboratory  
25 strain of HIV-1, NL4-3. Genotypes and corresponding  
phenotypes (fold-change in IC50 values) are entered in a  
relational database linking these two results with patient  
information. Large datasets can then be assembled from  
patient virus samples sharing particular characteristics,  
30 such as the presence of any given mutation or reduced  
susceptibility to any drug or combination of drugs.

**Protease inhibitor susceptibility of viruses containing  
mutations at amino acids 82 and 90 of HIV-1 Protease.**

35 Phenotypic susceptibility profiles of 33 patient virus  
samples which contained mutations at positions 82 (V82A,

5 F, S, or T) and 90 (L90M), but no other primary mutations,  
were analyzed. According to most published guidelines,  
such viruses are expected to be resistant to ritonavir,  
nelfinavir, indinavir, and saquinavir. However, 9% and  
21% of these samples were phenotypically susceptible to  
10 indinavir and saquinavir, respectively (see Table 6).  
Thus, particularly for saquinavir, there was poor  
correlation between the presence of mutations at positions  
82 and 90 and drug susceptibility.

15

#### EXAMPLE 10

#### Measuring Replication Fitness Using Resistance Test 20 Vectors

A means and method is provided for accurately measuring  
and reproducing the replication fitness of HIV-1. This  
method for measuring replication fitness is applicable to  
25 other viruses, including, but not limited to  
hepadnaviruses (human hepatitis B virus), flaviviruses  
(human hepatitis C virus) and herpesviruses (human  
cytomegalovirus). This example further provides a means  
and method for measuring the replication fitness of HIV-1  
30 that exhibits reduced drug susceptibility to reverse  
transcriptase inhibitors and protease inhibitors. This  
method can be used for measuring replication fitness for  
other classes of inhibitors of HIV-1 replication,  
including, but not limited to integration, virus assembly,  
35 and virus attachment and entry.

5     Replication fitness tests are carried out using the means  
and methods for phenotypic drug susceptibility and  
resistance tests described in US Patent Number 5,837,464  
(International Publication Number WO 97/27319) which is  
hereby incorporated by reference.

10

In these experiments patient-derived segment(s)  
corresponding to the HIV protease and reverse  
transcriptase coding regions were either patient-derived  
segments amplified by the reverse transcription-polymerase  
15 chain reaction method (RT-PCR) using viral RNA isolated  
from viral particles present in the serum of HIV-infected  
individuals or were mutants of wild type HIV-1 made by  
site directed mutagenesis of a parental clone of  
resistance test vector DNA. Resistance test vectors are  
20 also referred to as "fitness test vectors" when used to  
evaluate replication fitness. Isolation of viral RNA was  
performed using standard procedures (e.g. RNeasy Total  
RNA Isolation System, Promega, Madison WI or RNeasy,  
Tel-Test, Friendswood, TX). The RT-PCR protocol was  
25 divided into two steps. A retroviral reverse  
transcriptase [e.g. Moloney MuLV reverse transcriptase  
(Roche Molecular Systems, Inc., Branchburg, NJ), or avian  
myeloblastosis virus (AMV) reverse transcriptase,  
(Boehringer Mannheim, Indianapolis, IN)] was used to copy  
30 viral RNA into cDNA. The cDNA was then amplified using a  
thermostable DNA polymerase [e.g. Taq (Roche Molecular  
Systems, Inc., Branchburg, NJ), Tth (Roche Molecular  
Systems, Inc., Branchburg, NJ), PrimeZyme (isolated from  
Thermus brockianus, Biometra, Gottingen, Germany)] or a  
35 combination of thermostable polymerases as described for  
the performance of "long PCR" (Barnes, W.M., (1994) Proc.



5 Natl. Acad. Sci, USA 91, 2216-2220) [e.g. Expand High Fidelity PCR System (Taq + Pwo), (Boehringer Mannheim. Indianapolis, IN) OR GeneAmp XL PCR kit (Tth + Vent), (Roche Molecular Systems, Inc., Branchburg, NJ)].

10 PCR6 (Table 5, #1) is used for reverse transcription of viral RNA into cDNA. The primers, ApaI primer (PDSApa, Table 5, #2) and AgeI primer (PDSAge, Table 5, #3) used to amplify the "test" patient-derived segments contained sequences resulting in ApaI and AgeI recognition sites  
15 being introduced into both ends of the PCR product, respectively.

Fitness test vectors incorporating the "test" patient-derived segments were constructed as described in  
20 US Patent Number 5,837,464 (International Publication Number WO 97/27319) (see Fig. 1) using an amplified DNA product of 1.5 kB prepared by RT-PCR using viral RNA as a template and oligonucleotides PCR6 (#1), PDSApa (#2) and  
25 PDSAge (#3) as primers, followed by digestion with ApaI and AgeI or the isoschizomer PinAI. To ensure that the plasmid DNA corresponding to the resultant fitness test vector comprises a representative sample of the HIV viral quasi-species present in the serum of a given patient,  
30 many (>100) independent E. coli transformants obtained in the construction of a given fitness test vector were pooled and used for the preparation of plasmid DNA.

A packaging expression vector encoding an amphotrophic  
35 MuLV 4070A env gene product enables production in a fitness test vector host cell of fitness test vector viral

5 particles which can efficiently infect human target cells.  
Fitness test vectors encoding all HIV genes with the  
exception of env were used to transfect a packaging host  
cell (once transfected the host cell is referred to as a  
fitness test vector host cell). The packaging expression  
10 vector which encodes the amphotrophic MuLV 4070A env gene  
product is used with the resistance test vector to enable  
production in the fitness test vector host cell of  
infectious pseudotyped fitness test vector viral  
particles.

15 Fitness tests performed with fitness test vectors were  
carried out using packaging host and target host cells  
consisting of the human embryonic kidney cell line 293  
(Cell Culture Facility, UC San Francisco, SF, CA)..

20 Fitness tests were carried out with fitness test vectors  
using two host cell types. Fitness test vector viral  
particles were produced by a first host cell (the fitness  
test vector host cell) that was prepared by transfecting a  
25 packaging host cell with the fitness test vector and the  
packaging expression vector. The fitness test vector  
viral particles were then used to infect a second host  
cell (the target host cell) in which the expression of the  
indicator gene is measured (see Fig. A).

30 The fitness test vectors containing a functional  
luciferase gene cassette were constructed and host cells  
were transfected with the fitness test vector DNA. The  
fitness test vectors contained patient-derived reverse  
35 transcriptase and protease DNA sequences that encode  
proteins which were either susceptible or resistant to the

5     antiretroviral agents, such as nucleoside reverse  
transcriptase inhibitors, non-nucleoside reverse  
transcriptase inhibitors and protease inhibitors.\_

10     The amount of luciferase activity detected in the infected  
cells is used as a direct measure of "infectivity",  
"replication capacity" or "fitness", i.e. the ability of  
the virus to complete a single round of replication.  
Relative fitness is assessed by comparing the amount of  
luciferase activity produced by patient derived viruses to  
15     the amount of luciferase activity produced by a well-  
characterized reference virus (wildtype) derived from a  
molecular clone of HIV-1, for example NL4-3 or HXB2.  
Fitness measurements are expressed as a percent of the  
reference, for example 25%, 50%, 75%, 100% or 125% of  
20     reference (Figure B, C).

Host cells were seeded in 10-cm-diameter dishes and were  
transfected one day after plating with fitness test vector  
plasmid DNA and the envelope expression vector.  
25     Transfections were performed using a calcium-phosphate  
co-precipitation procedure. The cell culture media  
containing the DNA precipitate was replaced with fresh  
medium, from one to 24 hours, after transfection. Cell  
culture media containing fitness test vector viral  
30     particles was harvested one to four days after  
transfection and was passed through a 0.45-mm filter  
before being stored at -80°C. HIV capsid protein (p24)  
levels in the harvested cell culture media were determined  
by an EIA method as described by the manufacturer (SIAC;  
35     Frederick, MD). Before infection, target cells (293 and  
293/T) were plated in cell culture media. Control

5 infections were performed using cell culture media from  
mock transfections (no DNA) or transfections containing  
the fitness test vector plasmid DNA without the envelope  
expression plasmid. One to three or more days after  
infection the media was removed and cell lysis buffer  
10 (Promega) was added to each well. Cell lysates were  
assayed for luciferase activity. Alternatively, cells  
were lysed and luciferase was measured by adding Steady-  
Glo (Promega) reagent directly to each well without  
aspirating the culture media from the well.

15

#### **Example 11**

##### **Measuring Replication Fitness of Viruses with Deficiencies in Reverse Transcriptase Activity**

20 A means and method is provided for identifying mutations  
in reverse transcriptase that alter replication fitness.  
A means and method is provided for identifying mutations  
that alter replication fitness and can be used to identify  
mutations associated with other aspects of HIV-1  
25 replication, including, but not limited to integration,  
virus assembly, and virus attachment and entry. This  
example also provides a means and method for quantifying  
the affect that specific mutations reverse transcriptase  
have on replication fitness. A means and method for  
30 quantifying the affect that specfic protease and reverse  
transcriptase mutations have on replication fitness to  
mutations in other viral genes involved in HIV-1  
replication, including, but not limited to the gag, pol,  
and envelope genes is also provided.

35

Fitness test vectors were constructed as described in

5 example 10. Fitness test vectors derived from patient  
samples or clones derived from the fitness test vector  
pools, or fitness test vectors were engineered by site  
directed mutagenesis to contain specific mutations, and  
were tested in a fitness assay to determine accurately and  
10 quantitatively the relative fitness compared to a well-  
characterized reference standard. A patient sample was  
examined for increased or decreased reverse transcriptase  
activity and correlated with the relative fitness observed  
(Figure C).

15 **Reverse transcriptase activity of patient HIV samples**

Reverse transcriptase activity can be measured by any  
number of widely used assay procedures, including but not  
limited to homopolymeric extension using (e.g. oligo  
dT:poly rC) or real time PCR based on molecular beacons  
20 (reference Kramer) or 5' exonuclease activity (Lie and  
Petropoulos, 1996). In one embodiment, virion associated  
reverse transcriptase activity was measured using a  
quantitative PCR assay that detects the 5' exonuclease  
activity associated with thermo-stable DNA polymerases  
25 (Figure C). In one embodiment of the invention, the  
fitness of the patient virus was compared to a reference  
virus to determine the relative fitness compared to  
"wildtype" viruses that have not been exposed to reverse  
transcriptase inhibitor drugs. In another embodiment, the  
30 fitness of the patient virus was compared to viruses  
collected from the same patient at different timepoints,  
for example prior to initiating therapy, before or after  
changes in drug treatment, or before or after changes in  
virologic (RNA copy number), immunologic (CD4 T-cells), or  
35 clinical (opportunistic infection) markers of disease  
progression.

5

**Genotypic analysis of patient HIV samples**

10 Fitness test vector DNAs, either pools or clones, are analyzed by any of the genotyping methods described in Example 1. In one embodiment of the invention, patient HIV sample sequences were determined using viral RNA purification, RT/PCR and ABI chain terminator automated sequencing. The sequence was determined and compared to reference sequences present in the database or compared to a sample from the patient prior to initiation of therapy.

15 The genotype was examined for sequences that are different from the reference or pre-treatment sequence and correlated to the observed fitness.

**Fitness analysis of site directed mutants**

20

Genotypic changes that are observed to correlate with changes in fitness were evaluated by construction of fitness vectors containing the specific mutation on a defined, wild-type (drug susceptible) genetic background.

25 Mutations may be incorporated alone and/or in combination with other mutations that are thought to modulate the fitness of a virus. Mutations were introduced into the fitness test vector through any of the widely known methods for site-directed mutagenesis. In one embodiment

30 of this invention the mega-primer PCR method for site-directed mutagenesis is used. A fitness test vector containing the specific mutation or group of mutations were then tested using the fitness assay described in Example 10 and the fitness was compared to that of a

35 genetically defined wild-type (drug susceptible) fitness test vector which lacks the specific mutations. Observed

5 changes in fitness are attributed to the specific  
mutations introduced into the resistance test vector. In  
several related embodiments of the invention, fitness test  
vectors containing site directed mutations in reverse  
transcriptase that result in amino acid substitutions at  
10 position 190 (G190A, G190S, G190C, G190E, G190V, G190T)  
and that display different amounts of reverse  
transcriptase activity were constructed and tested for  
fitness (Figure D). The fitness results were correlated  
with specific reverse transcriptase amino acid  
15 substituions and fitness.

#### **Example 12**

##### **Measuring Replication Fitness of Viruses with Deficiencies in Protease Activity**

20 A means and method for identifying mutations in protease  
that alter replication fitness is provided.

This example provides the means and methods for  
25 identifying mutations that alter replication fitness for  
various components of HIV-1 replication, including, but  
not limited to integration, virus assembly, and virus  
attachment and entry. This example also provides a means  
and method for quantifying the affect that specific  
30 mutations in protease or reverse transcriptase have on  
replication fitness. This method can be used for  
quantifying the effect that specific protease mutations  
have on replication fitness and can be used to quantify  
the effect of other mutations in other viral genes  
35 involved in HIV-1 replication, including, but not limited  
to the gag, pol, and envelope genes.



5

Fitness test vectors were constructed as described in example 10. Fitness test vectors derived from patient samples or clones derived from the fitness test vector pools, or fitness test vectors engineered by site directed mutagenesis to contain specific mutations, were tested in a fitness assay to determine accurately and quantitatively the relative fitness compared to a well-characterized reference standard. A patient sample was examined further for increased or decreased protease activity correlated with the relative fitness observed (Figure C).

15

#### **Protease activity of patient HIV samples**

Protease activity can be measured by any number of widely used assay procedures, including but not limited to in vitro reactions that measure protease cleavage activity (reference Erickson ). In one embodiment, protease cleavage of the gag polyprotein (p55) was measured by Western blot analysis using an anti-capsid (p24) antibody (Figure C). In one embodiment of the invention, the fitness of the patient virus was compared to a reference virus to determine the relative fitness compared to "wildtype" viruses that have not been exposed to protease inhibitor drugs. In another embodiment, the fitness of the patient virus was compared to viruses collected from the same patient at different timepoints, for example prior to initiating therapy, before or after changes in drug treatment, or before or after changes in virologic (RNA copy number), immunologic (CD4 T-cells), or clinical (opportunistic infection) markers of disease progression.

35

#### **Genotypic analysis of patient HIV samples**

5 Fitness test vector DNAs, either pools or clones, are  
analyzed by any of the genotyping methods described in  
Example 1. In one embodiment of the invention, patient  
HIV sample sequences were determined using viral RNA  
purification, RT/PCR and ABI chain terminator automated  
10 sequencing. The sequence was determined and compared to  
reference sequences present in the database or compared to  
a sample from the patient prior to initiation of therapy,  
if available. The genotype was examined for sequences  
that are different from the reference or pre-treatment  
15 sequence and correlated to the observed fitness.

#### **Fitness analysis of site directed mutants**

Genotypic changes that are observed to correlate with  
20 changes in fitness are evaluated by construction of  
fitness vectors containing the specific mutation on a  
defined, wild-type (drug susceptible) genetic background.  
Mutations may be incorporated alone and/or in combination  
with other mutations that are thought to modulate the  
25 fitness of a virus. Mutations are introduced into the  
fitness test vector through any of the widely known  
methods for site-directed mutagenesis. In one embodiment  
of this invention the mega-primer PCR method for  
site-directed mutagenesis is used. A fitness test vector  
30 containing the specific mutation or group of mutations are  
then tested using the fitness assay described in Example  
10 and the fitness is compared to that of a genetically  
defined wild-type (drug susceptible) fitness test vector  
which lacks the specific mutations. Observed changes in  
35 fitness are attributed to the specific mutations  
introduced into the fitness test vector. In several

5 related embodiments of the invention, fitness test vectors  
containing site directed mutations in reverse protease  
that result in amino acid substitutions at positions 30,  
63, 77, 90 (list from Figure E) and that display different  
amounts of protease activity are constructed and tested  
10 for fitness (Figure E). The fitness results enable the  
correlation between specific protease amino acid  
substitutions and changes in viral fitness.

### Example 13

#### 15 **Measuring Replication Fitness and Drug Susceptibility in a Large Patient Population**

This example describes the high incidence of patient  
samples with reduced replication fitness. This example  
20 also describes the general correlation between reduced  
drug susceptibility and reduced replication fitness. This  
example further describes the occurrence of viruses with  
reduced fitness in patients receiving protease inhibitor  
and/or reverse transcriptase inhibitor treatment. This  
25 example further describes the incidence of patient samples  
with reduced replication fitness in which the reduction in  
fitness is due to altered protease processing of the gag  
polyprotein (p55). This example further describes the  
incidence of protease mutations in patient samples that  
30 exhibit low, moderate or normal (wildtype) replication  
fitness. This example further describes protease mutations  
that are frequently observed, either alone or in  
combination, in viruses that exhibit reduced replication  
capacity. This example also describes the incidence of  
35 patient samples with reduced replication fitness in which  
the reduction in fitness is due to altered reverse

5 transcriptase activity. This example describes the occurrence of viruses with reduced replication fitness in patients failing antiretroviral drug treatment.

10 Fitness/resistance test vectors were constructed as described in example 10. Fitness and drug susceptibility was measured in 134 random patient samples that were received for routing phenotypic testing by the ViroLogic Clinical Reference Laboratory. Fitness assays were performed as described in Example 10. Drug susceptibility testing and genotyping of the protease region was performed as described in Example 1. Reverse transcriptase activity was measured as described in Example 11. Protease processing was measured as described in Example 12.

20

#### **Drug susceptibility of patient viruses**

Reduced drug susceptibility was observed for a majority of the patient virus samples (Table A). 66 percent of the viruses exhibited large (define as >10X of the reference) reductions in susceptibility to one or more NRTI drugs. 52 percent of the viruses exhibited large reductions in susceptibility to one or more NNRTI drugs. 45 percent of the viruses exhibited large reductions in susceptibility to one or more PRI drugs.

30

#### **Fitness of patient viruses**

Reduced replication fitness was observed for a majority of the patient virus samples (Table A). Forty one percent of the viruses exhibited large reductions in replication fitness (<25% of the reference). Another 45% had moderate reductions (between 25-75% of the reference) in

35

5 replication fitness. A minority of the patient samples  
(14%) displayed replication fitness that approached or  
exceeded "wildtype" levels (>75% of the reference).  
Viruses with reduced drug susceptibility, were much more  
likely to display reduced replication fitness (Figures F,  
10 G, H, and I).

#### **Protease Mutations in patient viruses**

Greater than 10 mutations in protease were observed in a  
majority of the patient virus samples (Table A). Viruses  
15 with reduced fitness were much more likely to contain 10  
or more protease mutations (Figure I). Sixty two percent  
of the viruses that exhibited large reductions in  
replication fitness (<25% of the reference) contained 10  
or more protease mutations. Twenty two percent of the  
20 viruses with moderate reductions (between 25-75% of the  
reference) in fitness contained 10 or more protease  
mutations. Only 5% of the viruses that displayed  
replication fitness that approached or exceeded "wildtype"  
levels (>75% of the reference) contained 10 or more  
25 protease mutations (Table A). Certain protease mutations  
either alone (D30N) or in combination (L90M plus K20T, or  
M46I, or 73, or N88D) were observed at high incidences in  
viruses with reduced fitness (Figures I and J).

#### **Protease processing of patient viruses**

30 Reduced protease processing of the p55 gag polyprotein was  
observed in a majority of the patient virus samples (Table  
A). Viruses with reduced fitness were much more likely to  
display reduced protease processing; defined as having

5 detectable amounts of the p41 intermediate cleavage  
product (Figures F, I and K). Seventy one percent of the  
viruses that exhibited large reductions in replication  
fitness (<25% of the reference) displayed reduced protease  
processing. Eighteen percent of the viruses with moderate  
10 fitness reductions (between 25-75% of the reference)  
displayed reduced protease processing. Only 10% of the  
viruses that displayed replication fitness that approached  
or exceeded "wildtype" levels (>75% of the reference)  
exhibited reduced protease processing (Table A). Certain  
15 protease mutations (D30N, M46I/L, G48V, I54L/A/S/T/V, and  
I84V) were observed at high incidences in viruses with  
reduced protease processing of the p55 gag polyprotein  
(Figure L).

20 **Reverse transcriptase of patient viruses**

Reduced reverse transcriptase activity processing was  
observed in a minority of the patient virus samples (Table  
A). Viruses with reduced fitness were much more likely to  
display reduced reverse transcriptase activity. Fourteen  
25 percent of the viruses that exhibited large reductions in  
replication fitness (<25% of the reference) displayed  
reduced reverse transcriptase activity. Only 2% of the  
viruses with moderate fitness reductions (between 25-75%  
of the reference) displayed reduced reverse transcriptase  
30 activity. None of the viruses that displayed replication  
fitness that approached or exceeded "wildtype" levels  
(>75% of the reference) exhibited reduced reverse  
transcriptase activity.

5

#### Example 14

#### Measuring Replication Fitness to Guide Treatment Decisions

A means and method for using replication fitness measurements to guide the treatment of HIV-1 is provided. This example further provides a means and method for using replication fitness measurements to guide the treatment of patients failing antiretroviral drug treatment. This example further provides the means and methods for using replication fitness measurements to guide the treatment of patients newly infected with HIV-1.

**Guiding treatment of patients with multi-drug resistant virus:** Fitness/resistance test vectors were constructed as described in example 10. Fitness and drug susceptibility were measured on serial longitudinal samples collected weekly for 12 weeks from 18 patients. These patients were considered failing a protease inhibitor (typically indinavir) containing regimen and had incomplete suppression of virus replication based on routine viral load testing ( $>2,500$  copies/mL). Phenotypic drug susceptibility testing indicated that these patient viruses were multi-drug resistant. Each patient agreed to interrupt therapy for a period of at least 12 weeks. Phenotypic drug susceptibility assays were performed as described in Example 1 on serial samples collected just prior to interrupting therapy and weekly during the period of interruption. Fitness assays were performed as described in Example 10 on serial samples collected just prior to interrupting therapy and weekly during the period



5 of interruption. Protease processing was measured as described in Example 12.

10 Of the 18 patients that interrupted therapy, 16 patients had resistant viruses that regained susceptibility to antiretroviral drugs during the period of treatment interruption. The phenotypic test results of a representative patient are shown in Figure M. Typically, susceptibility returned to all drug classes simultaneously, consistent with the re-emergence of a  
15 minor population of drug sensitive virus. In the representative example shown in Figure M, drug sensitivity was abruptly restored between weeks 9 and 10. Genotypic analysis (DNA sequence of protease and reverse transcriptase) are also consistent with the re-emergence  
20 of a drug sensitive virus. These data show the loss of most or all drug resistance mutation simultaneously (data not shown). The data are not consistent with random back mutations. Back mutations would predict that restored susceptibility to drugs would occur unevenly for different  
25 drug classes and/or within a drugs within the same class.

Generally, the re-emergence of the drug susceptible virus was also accompanied by a simultaneous increase in replication fitness. This relationship is clearly evident  
30 for the representative virus (Figure N). Several other examples with less frequent timepoints are shown in Figure O. Virus from patients that did not revert to drug susceptibility after interruption generally did not exhibit an increase in replication fitness, nor did

5 viruses from patients that did not interrupt treatment  
(Figures 0). The data indicate that the drug sensitive  
virus that re-emerged after treatment interruption is able  
to replicate better than the drug resistant virus that was  
present before treatment was interrupted. The re-  
10 emergence of drug susceptible virus in this group of  
patients was also accompanied by an increase in viral load  
and a decrease in DC4 T-cells, indicators of disease  
progression. Thus, fitness information can be used to  
guide treatment of patients that harbor multi-drug  
15 resistant virus and are considering treatment  
interruption. If the patient virus is drug resistant but  
has low replication capacity, the patient and the  
physician should consider continuing drug treatment to  
prevent the re-emergence of a drug sensitive virus with  
20 higher replication capacity and greater pathogenicity.  
Alternatively, if the patient virus is drug resistant and  
has high replication capacity, the patient and the  
physician may consider interrupting treatment to spare the  
patient from the harmful and unpleasant side effects of  
25 antiretroviral drugs that are not providing clinical  
benefit.

Furthermore, physicians may choose to perform routine  
replication fitness assays for patients that have multi-  
30 drug resistant virus. This assay could be used to monitor  
the replication fitness of patient viruses when complete  
suppression of virus replication is not possible due to  
multi-drug resistance. The assay would be used to guide  
treatment decisions that prevent the drug resistant virus

5 with low replication fitness from increasing its  
replication fitness. In this way, physicians may prolong  
the usefulness of antiretroviral drugs despite the  
presence of drug resistant virus in the patient.

10 **Guiding treatment of newly infected patients:**

15 Patients that maintain high virus loads (setpoint) after  
acute infection are more likely to exhibit accelerated  
disease progression. Therefore, it is advantageous for  
this class of patient to initiate antiretroviral drug  
treatment as soon as possible after diagnosis with HIV-1  
infection. In conjunction with viral load, fitness  
measurements of viruses in newly infected patients may  
provide a useful measurement to identify those individuals  
20 that will develop elevated setpoints after primary  
infection and consequently are likely to exhibit  
accelerated disease progression. Fitness measurements may  
guide the decision to treat immediately after diagnosis or  
a some later time point.

25

**Example 15**

**Measuring Saquinavir Susceptibility of Viruses  
Containing Various Amino Acid Substitutions in  
Protease at Position 82**

30

This example provides a means and method for identifying  
mutations in protease that affect susceptibility  
(increased or decreased) to saquinavir.

5 In one embodiment of this invention, the effects of  
combination of mutations at position 82 (for example,  
V82A, V82F, V82S, or V82T are evaluated using the  
following method comprising: (i)collecting a biological  
10 whether the HIV-1 in the sample contains nucleic acid  
encoding protease having a valine to alanine (V82A),  
phenylalanine (V82F), serine (V82S), or threonine (V82T)  
substitution at position 82 or a leucine to methionine  
substitution at position 90 (L90M); and (iii) determining  
15 susceptibility to protease inhibitors (PRIs).

The biological sample comprises whole blood, blood  
components including peripheral mononuclear cells (PBMC),  
serum, plasma (prepared using various anticoagulants such  
20 as EDTA, acid citrate-dextrose, heparin), tissue biopsies,  
cerebral spinal fluid (CSF), or other cell, tissue or body  
fluids. In another embodiment, the HIV-1 nucleic acid  
(genomic RNA) or reverse transcriptase protein can be  
isolated directly from the biological sample or after  
25 purification of virus particles from the biological  
sample. Evaluating whether the amino acid at position 82  
of the HIV-1 protease is mutated to alanine,  
phenylalanine, or threonine, can be performed using  
various methods, such as direct characterization of the  
30 viral nucleic acid encoding protease or direct  
characterization of the protease protein itself. Defining  
the amino acid at position 82 of protease can be performed  
by direct characterization of the protease protein by  
conventional or novel amino acid sequencing methodologies,

5 epitope recognition by antibodies or other specific  
binding proteins or compounds. Alternatively, the amino  
acid at position 82 of the HIV-1 protease protein can be  
defined by characterizing amplified copies of HIV-1  
10 nucleic acid encoding the protease protein. Amplification  
of the HIV-1 nucleic acid can be performed using a variety  
of methodologies including reverse  
transcription-polymerase chain reaction (RT-PCR), NASBA,  
SDA, RCR, or 3SR. The nucleic acid sequence encoding HIV  
15 protease at codon 82 can be determined by direct nucleic  
acid sequencing using various primer extension-chain  
termination (Sanger, ABI/PE and Visible Genetics) or chain  
cleavage (Maxam and Gilbert) methodologies or more  
recently developed sequencing methods such as matrix  
20 assisted laser desorption-ionization time of flight  
(MALDI-TOF) or mass spectrometry (Sequenom, Gene Trace  
Systems). Alternatively, the nucleic acid sequence  
encoding amino acid position 82 can be evaluated using a  
variety of probe hybridization methodologies, such as  
25 genechip hybridization sequencing (Affymetrix), line probe  
assay (LiPA; Murex), and differential hybridization  
(Chiron).

30 In a preferred embodiment of this invention, evaluation of  
the effects of mutations at amino acid position 82 of  
HIV-1 protease on protease inhibitor susceptibility, was  
carried out using a phenotypic susceptibility assay using  
resistance test vector DNA prepared from the biological  
sample. In one embodiment, plasma samples were collected,

5 viral RNA was purified and an RT-PCR methodology was used  
to amplify a patient derived segment encoding the HIV-1  
protease and reverse transcriptase regions. The amplified  
patient derived segments were then incorporated, via DNA  
ligation and bacterial transformation, into an indicator  
10 gene viral vector thereby generating a resistance test  
vector. Resistance test vector DNA was isolated from the  
bacterial culture and the phenotypic susceptibility assay  
was carried out as described in Example 1. The genotype of  
the protease region was determined by dideoxy chain-  
15 termination sequencing of the resistance test vector DNA.  
The results are summarized for saquinavir (SQV) in Figure  
6. Samples were categorized as having mutations in  
protease encoding alanine (A), phenylalanine (F), or  
threonine (T) at position 82, instead of the wild-type  
20 valine (V), and the percentage of samples in each category  
displaying hyper-sensitivity to saquinavir (i.e., fold-  
change vs. reference of 0.4 or less) was determined.  
Surprisingly, the percentage of saquinavir hyper-  
susceptible viruses was much higher amongst viruses  
25 containing V82F than those containing V82A or V82T. This  
observation implies that the detection of V82F in protease  
predicts a positive virological response to saquinavir  
treatment.

5

### Example 16

#### Measuring Replication Fitness of Viruses with Mutations in Integrase

10      This example provides a means and method for identifying  
mutations in integrase that alter replication fitness.

15      This example provides the means and methods for  
identifying mutations that alter replication fitness for  
various components of HIV-1 replication, including, but  
not limited to integration, virus assembly, and virus  
attachment and entry. This example also provides a means  
and method for quantifying the affect that specific  
mutations in protease, reverse transcriptase, or integrase  
20      have on replication fitness. This method can be used for  
quantifying the effect that specific integrase mutations  
have on replication fitness and can be used to quantify  
the effect of other mutations in other viral genes  
involved in HIV-1 replication, including, but not limited  
25      to the gag, pol, and envelope genes.

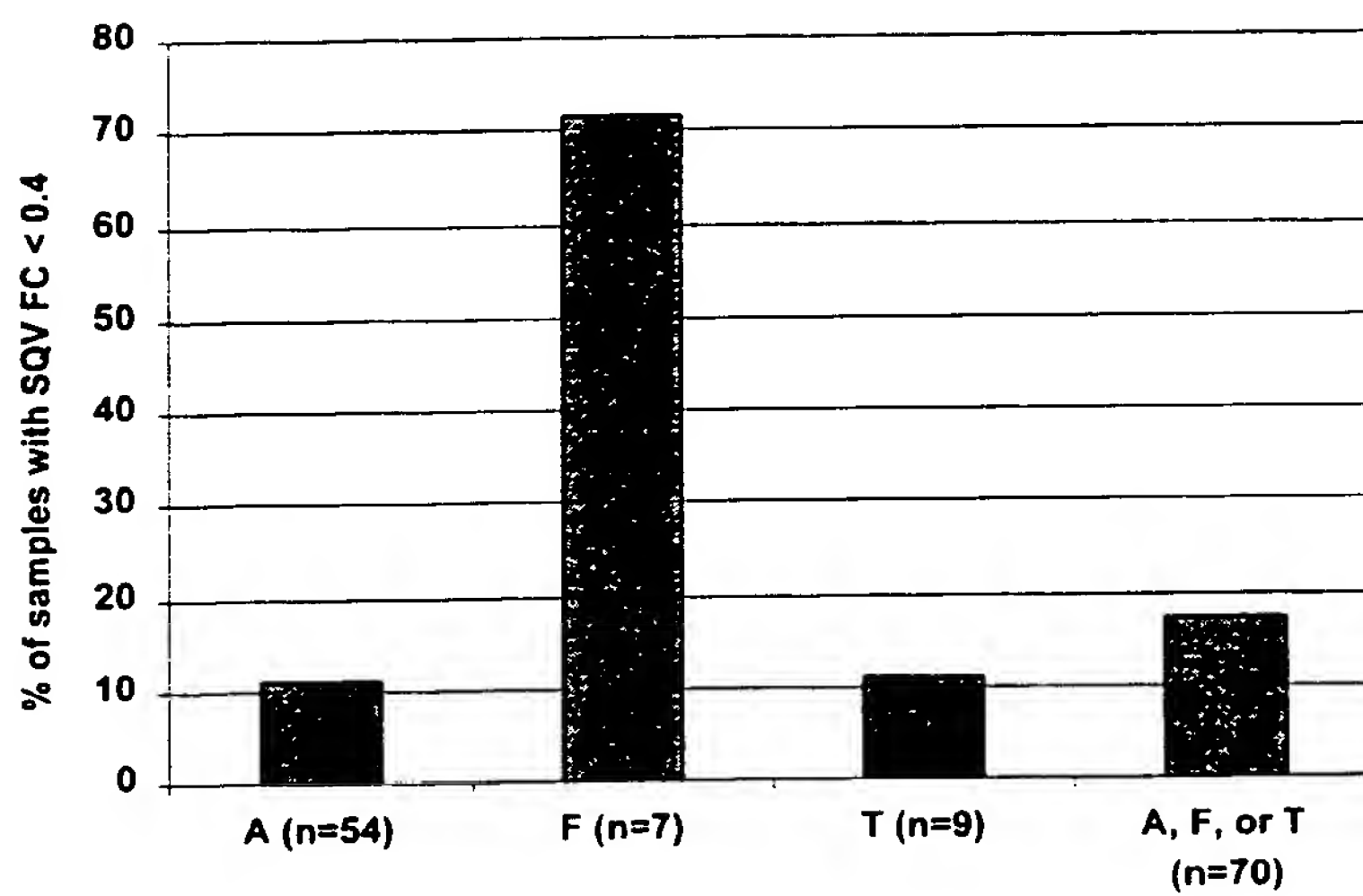
30      Fitness test vectors engineered by site directed  
mutagenesis to contain specific mutations in integrase  
were tested in a fitness assay to determine accurately and  
quantitatively the relative fitness compared to a well-  
characterized reference standard.

Genotypic changes that are observed to correlate with  
resistance to integrase inhibitors are evaluated by



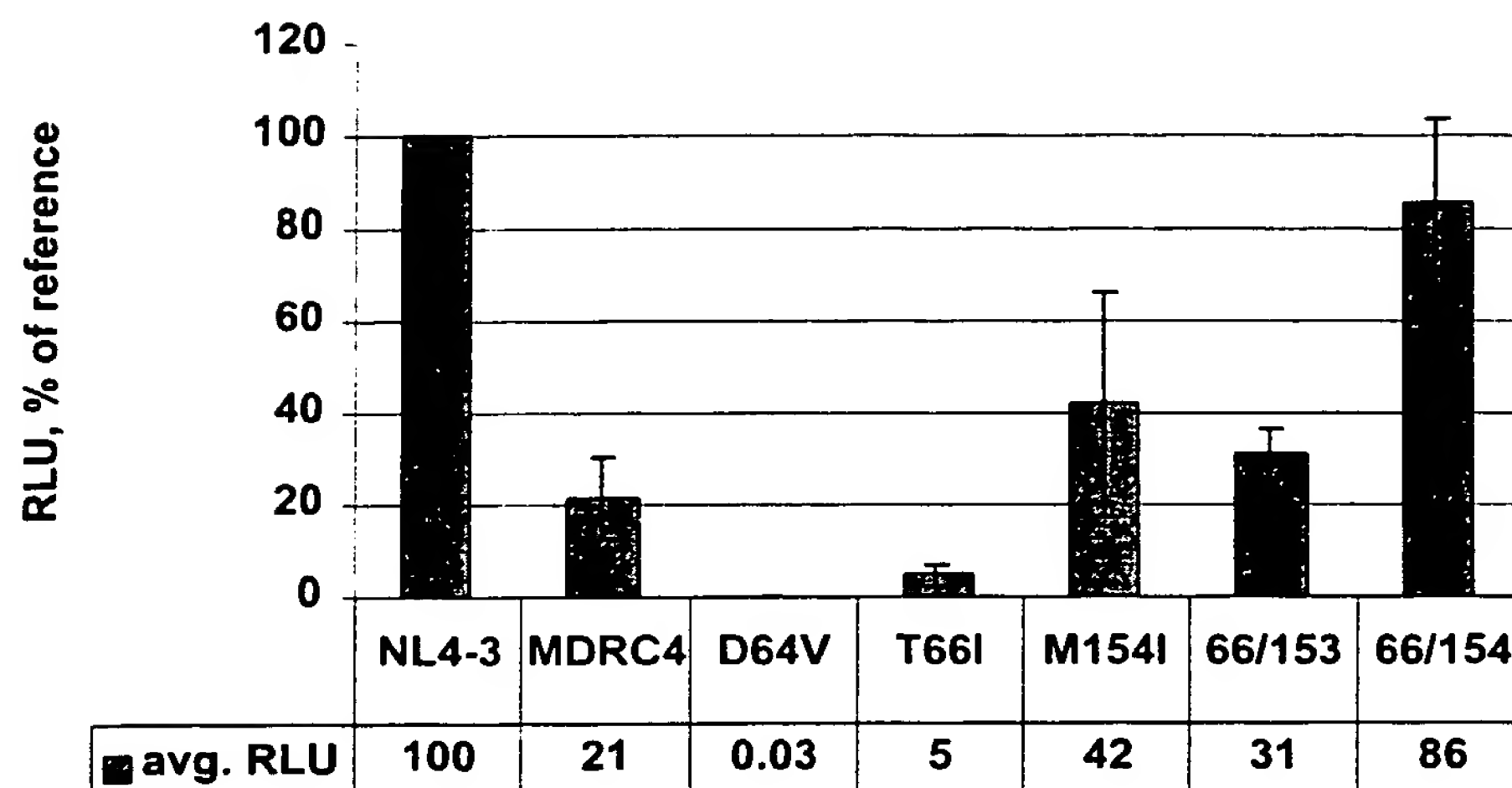
5 construction of fitness vectors containing the specific  
mutation on a defined, wild-type (drug susceptible)  
genetic background. Mutations may be incorporated alone  
and/or in combination with other mutations that are  
10 thought to modulate the fitness of a virus. Mutations  
are introduced into the fitness test vector through any of  
the widely known methods for site-directed mutagenesis. In  
one embodiment of this invention the mega-primer PCR  
method for site-directed mutagenesis is used (Sarkar, G.  
and Sommer, S.S., 1994, Biotechniques 8, 404-407). A  
15 fitness test vector containing the specific mutation or  
group of mutations are then tested using the fitness assay  
described in Example 10 and the fitness is compared to  
that of a genetically defined wild-type (drug susceptible)  
fitness test vector which lacks the specific mutations.  
20 Observed changes in fitness are attributed to the specific  
mutations introduced into the fitness test vector. In  
several related embodiments of the invention, fitness test  
vectors containing site directed mutations in integrase  
that result in amino acid substitutions at positions 66,  
25 154, 66 and 153, and 66 and 154 are constructed and tested  
for fitness (Figure 7). As controls, mutants with  
multiple changes conferring resistance to reverse  
transcriptase and protease inhibitors (MDRC4) and with a  
mutation in the integrase active site (D64V) were also  
30 tested. The fitness results enable the correlation  
between specific integrase amino acid substitutions and  
changes in viral fitness.

- 5 Figure 6. Distribution of saquinavir hyper-susceptibility by amino acid change at position 82.



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Figure 7. Relative luciferase activity of integrase inhibitor-resistant site-directed mutants.



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25 Table 1: PRI susceptibility of selected patient samples. Viruses displaying increased susceptibility to amprenavir (5-fold or greater) were genotyped and found to contain the N88S mutation in PR. Samples were listed in order of decreasing amprenavir susceptibility.

Table 1

| Sample ID | Prior PRI Experience | Fold Change vs. Reference |      |      |       |      | PR Mutations   |
|-----------|----------------------|---------------------------|------|------|-------|------|--|
|           |                      | SQV                       | IDV  | RTV  | NFV   | AMP  |  |
| 0732      | NFV                  | 0.73                      | 2.11 | 1.72 | 6.92  | 0.08 | K14R, I15V, K20T, E35D, M36I, R41K, I62V, L63Q, N88S |
| 627       | IDV                  | 0.26                      | 6.16 | 1.50 | 21.06 | 0.09 | I131V, E35D, M46L, L63P, I64V, I73V, N88S            |
| 1208      | NFV                  | 1.55                      | 3.15 | 1.22 | 11.06 | 0.10 | I62V, L63P, V77I, N88S                               |
| 360       | IDV                  | 1.88                      | 6.31 | 1.49 | 29.95 | 0.15 | I13V, K20M, M36V, N37A, M46I, I62V, L63P, N88S, I93L |
| 0910      | NFV                  | 1.41                      | 5.27 | 1.85 | 16.76 | 0.16 | M46I, L63P, V77I, N88S, I93I/L                       |
| 3542      | IDV                  | 1.28                      | 7.61 | 3.36 | 21.67 | 0.16 | I13V, K14R, N37D, M46I, L63P, N88S, I93L             |
| 3654      |                      | 1.80                      | 7.56 | 1.95 | 18.61 | 0.20 | I13V, R41K, M46I, L63P, V77I, N88S, I93L             |

Fold Change Limits: >2.5 <0.4

- 5 Table 2: PRI susceptibility of site-directed mutants in PR. Mutations were introduced into the drug sensitive reference resistance test vector and the susceptibility to PRIs was determined.

Table 2

| Site-Directed Mutations | Fold Change vs. reference |      |      |       |      |
|-------------------------|---------------------------|------|------|-------|------|
|                         | SQV                       | IDV  | RTV  | NFV   | AMP  |
| L63P                    | 1.04                      | 1.12 | 1.27 | 1.43  | 1.06 |
| L63P, V77I              | 1.24                      | 1.72 | 1.73 | 2.49  | 0.91 |
| N88S                    | 0.47                      | 1.56 | 0.36 | 2.39  | 0.04 |
| L63P, N88S              | 1.44                      | 2.56 | 0.77 | 5.10  | 0.11 |
| L63P, V77I, N88S        | 1.24                      | 3.09 | 1.39 | 12.69 | 0.08 |
| M46L, L63P, N88S        | 1.15                      | 2.30 | 0.85 | 6.16  | 0.12 |
| M46L, L63P, V77I, N88S  | 1.45                      | 2.97 | 1.33 | 12.24 | 0.14 |

FOLD CHANGE LIMITS: <0.4 >2.5

2020-04-29

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Table 3: Relative luciferase activity levels for patient sample virus-derived resistance test vector pools. The luciferase activity (relative light units, RLU) measured in the absence of drug for the patient sample was compared to that of the drug sensitive reference control from the same assay run, and expressed as a percentage of control. These values are from one assay each. All the samples that contain the N88S mutations in PR were found to have reduced luciferase activity compared to control.

Table 3

| Sample ID | PR Mutations   | Relative Luciferase Activity<br>(% of control) |
|-----------|--|--|
| 0732      | K14R, I15V, K20T, E35D, M36I, R41K, I62V, L63Q, N88S | 8.5  |
| 627       | I13I/V, E35D, M46L, L63P, I64V, I73V, N88S           | 0.7  |
| 1208      | I62V, L63P, V77I, N88S                               | 14.2   |
| 360       | I13V, K20M, M36V, N37A, M46I, I62V, L63P, N88S, I93L | 2.2  |
| 0910      | M46I, L63P, V77I, N88S, I93I/L                       | 16.0   |
| 3542      | I13V, K14R, N37D, M46I, L63P, N88S, I93L             | 4.6  |
| 3654      | I13V, R41K, M46I, L63P, V77I, N88S, I93L             | 12.8   |

5 Table 4: Relative luciferase activity levels for  
resistance test vectors containing site-directed  
mutations. The luciferase activity (relative light units,  
10 RLU) measured in the absence of drug for the mutant was  
compared to that of the drug sensitive reference control  
from the same assay run, and expressed as a percentage of  
control. These values are from one to five assays each,  
and each value was obtained using an independent clone for  
15 mutants which were tested multiple times. All the  
constructs that contain the N88S mutations in PR were  
found to have reduced luciferase activity compared to  
control. All the constructs with the K20T mutation were  
essentially inactive in the assay.

**Table 4**

| Site-Directed Mutations | Average Luciferase Activity |                         |
|-------------------------|-----------------------------|-------------------------|
|                         | (% of control)              | number of clones tested |
| L63P                    | 163.9                       | 1                       |
| L63P, V77I              | 75.6                        | 1                       |
| N88S                    | 1.0                         | 3                       |
| L63P, N88S              | 20.7                        | 2                       |
| L63P, V77I, N88S        | 29.3                        | 2                       |
| M46L, L63P, N88S        | 28.0                        | 2                       |
| M46L, L63P, V77I, N88S  | 53.2                        | 5                       |
| K20T, N88S              | <0.01                       | 5                       |
| K20T, L63P, N88S        | <0.01                       | 1                       |



5 Table 5: Oligonucleotide primers used for PCR  
amplification and for generating site-directed mutants.

10

**Table 5.**

| Primer name: |    |   |             |
|--------------|----|---|-------------|
| #1: PCR6     | 5' | CCAATTRYTGATATTTCTCATGNTCHTCTTGGG         | 3' (35-mer) |
| #2: PDS/Apa  | 5' | CATGTTGCAGGGCCCCTAGGAAAAGGGCTGTTGGAAATGTG | 3' (42-mer) |
| #3: PDS/Age  | 5' | CACTCCATGTACCGGTTCTTTAGAAATYTCYCTG        | 3' (34-mer) |
| #4: RsrII    | 5' | ACTTTCGGACCGTCCATTCTGGCTTTAATTTACTGGTACAG | 3' (43-mer) |
| #5: K20T     | 5' | GGGGGGCAATTAACGGAAGCTCTATTAG              | 3' (28-mer) |
| #6: M46L     | 5' | GATGGAAACCAAAATTGATAGGGGGAATTG            | 3' (30-mer) |
| #7: L63P     | 5' | GTATGATCAGATACCCATAGAAATCTGC              | 3' (28-mer) |
| #8: N88S     | 5' | CTGAGTCAACAGACTTCTTCCAATTATG              | 3' (28-mer) |

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R = A or G  
Y = C or T  
N = A, C, G, or T  
H = A, C, or T

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Table 6. PRI Susceptibility (Fold Change <2.5) of Viruses  
with Mutations at 82 and/or 90

| Percent of viruses with indicated primary<br>mutation(s) which are drug sensitive (fold<br>change in IC50 < 2.5) |            |      |                        |
|--|------------|------|------------------------|
| drug   | V82A/F/S/T | L90M | V82A/F/S/T and<br>L90M |
| RTV  | 8.0        | 27.6 | 3.0                    |
| NFV  | 20.0       | 8.6  | 3.0                    |
| IDV  | 22.7       | 31.0 | 9.1                    |
| AMP  | 53.3       | 65.5 | 33.3                   |
| SQV  | 73.3       | 46.6 | 21.2                   |

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**Table 7. Correlation Between 82A/F/S/T, Secondary Mutations, and IDV Susceptibility.**

| 10 | position | n  | % FC > 2.5 | chi square p |
|----|----------|----|------------|--------------|
|    | 24       | 20 | 100%       | <0.005       |
|    | 71       | 27 | 100%       | <0.0001      |
|    | 54       | 38 | 95%        | <0.0001      |
|    | 46       | 35 | 89%        | <0.01        |
| 15 | 10       | 47 | 83%        | <0.05        |
|    | 63       | 72 | 79%        | <0.05        |
|    | 82       | 75 | 77%        |              |

20 all virus with V82A/F/S/T and no other primary mutations.

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5 **Table 8. Correlation Between 82A/F/S/T, Secondary Mutations, and SQV Susceptibility.**

|    | position | n  | % FC > 2.5 | chi square p |
|----|----------|----|------------|--------------|
|    | 20       | 5  | 80%        | <0.001       |
|    | 36       | 11 | 73%        | <0.001       |
| 10 | 24       | 20 | 65%        | <0.0001      |
|    | 71       | 27 | 52%        | <0.0001      |
|    | 54       | 38 | 47%        | <0.0001      |
|    | 10       | 47 | 40%        | <0.001       |
| 15 | 82       | 75 | 27%        |              |

all virus

20 **Table 9. Association Between SQV and IDV Susceptibility, V82A/F/S/T, and Number of Resistance Associated Mutations**

|    | Number of secondary mutations | Number of samples | % with IDV FC > 2.5 | % with SQV FC > 2.5 |
|----|-------------------------------|-------------------|---------------------|---------------------|
| 25 | 1                             | 75                | 77                  | 27                  |
|    | 2                             | 67                | 82                  | 30                  |
|    | 3                             | 51                | 88                  | 39                  |
|    | 4                             | 38                | 95                  | 50                  |
| 30 | 5                             | 25                | 96                  | 60                  |
|    | 6                             | 17                | 100                 | 76                  |
|    | 7                             | 5                 | 100                 | 60                  |

10

15

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25

all viruses

5           **Table 12. Association Between SQV and IDV  
Susceptibility, L90M, and Number of Resistance Associated  
Mutations.**

|    | Number of<br>secondary<br>mutations | Number of<br>samples | % w with IDV<br>FC > 2.5 | % w<br>with SQV<br>FC > 2.5 |
|----|-------------------------------------|----------------------|--------------------------|-----------------------------|
| 10 | 0                                   | 58                   | 69                       | 53                          |
|    | 1                                   | 57                   | 70                       | 47                          |
|    | 2                                   | 56                   | 70                       | 48                          |
|    | 3                                   | 41                   | 80                       | 68                          |
| 15 | 4                                   | 31                   | 87                       | 77                          |
|    | 5                                   | 14                   | 100                      | 100                         |
|    | 6                                   | 6                    | 100                      | 100                         |

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For 2000-2009

**Summary of the Invention**

In another embodiment of this invention, a method is provided of assessing the effectiveness of protease antiretroviral therapy of an HIV-infected patient comprising:

- (a) collecting a biological sample from the HIV-infected patient;
- (b) evaluating whether the biological sample contains nucleic acid encoding HIV protease having a mutation at codon 82 and a secondary mutation at codons selected from the group consisting of 84, 48, 23, 73, 53, 33, 74, 20, 90, 32 and 39 or a mutation at codon 90 and a secondary mutation at codons selected from the group consisting of 53, 66, 84, 54, 48, 33, 73, 20, 71, 64 and 93, and
- (c) determining a change in susceptibility to a protease inhibitor, wherein the protease inhibitor is saquinavir.

In another embodiment of this invention, the above method is provided of assessing the effectiveness of protease antiretroviral therapy, having a mutation at codon 82 and a secondary mutation at codons selected from the group consisting of 84, 48, 23, 73, 53, 33, 74, 20, and 90, or a mutation at codon 90 and a secondary mutation at codons selected from the group consisting of 53, 66, 84, 54, 48, 33, 73, 20, and 71, wherein the change in susceptibility in step (c) is a decrease in susceptibility to saquinavir.

In another embodiment of this invention, the above method is provided of assessing the effectiveness of protease antiretroviral therapy, having a mutation at codon 82 and

a secondary mutation at codons 32 or 39, or a mutation at codon 90 and a secondary mutation at codons 64 or 93, wherein the change in susceptibility in step (c) is an increase in susceptibility to saquinavir.

5

In another embodiment of this invention, the above method is provided of assessing the effectiveness of protease antiretroviral therapy, having a mutation at codon 90 and a secondary mutation at codons selected from the group consisting of 53, 95, 54, 84, 82, 46, 13, and 74, wherein the protease inhibitor is indinavir.

10

In another embodiment of this invention, the above method is provided of assessing the effectiveness of protease antiretroviral therapy, having a mutation at codon 90 and a secondary mutation at codons selected from the group consisting of 53, 95, 54, 84, 82, and 46, wherein the change in susceptibility in step (c) is a decrease in susceptibility to indinavir.

15

20

In another embodiment of this invention, the above method is provided of assessing the effectiveness of protease antiretroviral therapy, having a mutation at codon 90 and a secondary mutation at codons 13 or 74, wherein the change in susceptibility in step (c) is an increase in susceptibility to indinavir.

25

In another embodiment of this invention, the above method is provided of assessing the effectiveness of protease antiretroviral therapy, having a mutation at codon 82 and a secondary mutation at codons selected from the group consisting of 73, 55, 48, 20, 43, 53, 90, 13, 48, 23, 84, 53, 74, 60, 33, 36, 35, 32, and 46 or a mutation at codon

30



90 and a secondary mutation at codons selected from the group consisting of 95, 55, 54, 82, 85, 84, 20, 72, 62, 74, 53, 48, 23, 58, 36, 64, 77, and 93.

5 In another embodiment of this invention, the above method is provided of assessing the effectiveness of protease antiretroviral therapy, wherein the protease inhibitor is selected from the group consisting of indinavir, amprenavir, and saquinavir.

10

In another embodiment of this invention, the above method is provided of assessing the effectiveness of protease antiretroviral therapy, wherein step (c) is determining a change in susceptibility to the protease inhibitor greater than 10 fold.

15

In another embodiment of this invention, the above method is provided of assessing the effectiveness of protease antiretroviral therapy, having a mutation at codon 82 and a secondary mutation at codons selected from the group consisting of 48, 23, 84, 53, 74, 20, 60, 33, 36, 35, or a mutation at codon 90 and a secondary mutation at codons selected from the group consisting of 84, 53, 48, 23, 58, 20, 36, and 54, wherein the change in susceptibility in step (c) is a decrease in susceptibility to saquinavir.

20

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In another embodiment of this invention, the above method is provided of assessing the effectiveness of protease antiretroviral therapy, having a mutation at codon 82 and a secondary mutation at codons 32 or 46, or a mutation at codon 90 and a secondary mutation at codons 64, 77, or 93, wherein the change in susceptibility in step (c) is an increase in susceptibility to saquinavir.

30

5 In another embodiment of this invention, the above method  
is provided of assessing the effectiveness of protease  
antiretroviral therapy, having a mutation at codon 82 and  
a secondary mutation at codons selected from the group  
consisting of 73, 55, 48, 20, 43, 53, and 90, or a  
mutation at codon 90 and a secondary mutation at codons  
selected from the group consisting of 95, 55, 54, 82, 85,  
84, 20, 72, and 62, wherein the change in susceptibility  
10 in step (c) is a decrease in susceptibility to indinavir.

In another embodiment of this invention, the above method  
is provided of assessing the effectiveness of protease  
antiretroviral therapy, having a mutation at codon 82 and  
a secondary mutation at codon 13, or a mutation at codon  
15 90 and a secondary mutation at codon 74, wherein the  
change in susceptibility in step (c) is an increase in  
susceptibility to indinavir.

20 In another embodiment of this invention, a method is  
provided of assessing the effectiveness of protease  
antiretroviral therapy of an HIV-infected patient  
comprising:

- (a) collecting a biological sample from the  
HIV-infected patient;
- 25 (b) evaluating whether the biological sample  
contains nucleic acid encoding HIV protease  
having a mutation at codon 90 and secondary  
mutations of at least three codons; and
- (c) determining a decrease in susceptibility to  
30 saquinavir.

In another embodiment of this invention, the above method  
is provided of assessing the effectiveness of protease

antiretroviral therapy, wherein in the evaluating step (b), the nucleic acid encoding HIV protease has secondary mutations of at least five codons.

5 In another embodiment of this invention, the above method is provided of assessing the effectiveness of protease antiretroviral therapy, wherein the secondary mutation are selected from the group consisting of codons 10, 20, 52, 53, 54, 66, 71, 73 and 84.

10

In another embodiment of this invention, a method is provided of assessing the effectiveness of protease antiretroviral therapy of an HIV-infected patient comprising:

15

(a) collecting a biological sample from the HIV-infected patient;

20

(b) evaluating whether the biological sample contains nucleic acid encoding HIV protease having a mutation at codon 82 and secondary mutations at codons selected from the group consisting of 33, 23, 84, 32, 53, 90, 37, 71, 46, 10, 54, 61, 11, and 46, or a mutation at codon 90 and secondary mutations at codons selected from the group consisting of 89, 53, 84, 33, 92, 95, 54, 58, 46, 82, 36, 10, 62, 74, 15, 47, 66, 32, 55, 53, 13, and 69; and

25

(c) determining a change in susceptibility to amprenavir.

30

In another embodiment of this invention, the above method is provided of assessing the effectiveness of protease antiretroviral therapy, wherein the mutation at codon 82 is a substitution of alanine (A), phenylalanine (F),

serine (S), or threonine (T) for valine(V) and the mutation at codon 90 is a substitution of methionine (M) for leucine (L).

5 In another embodiment of this invention, the above method is provided of assessing the effectiveness of protease antiretroviral therapy, having a mutation at codon 82 and secondary mutations at codons selected from the group consisting of 33, 23, 84, 32, 53, 90, 37, 71, 46, 10, 54,  
10 11, and 46, or a mutation at codon 90 and secondary mutations at codons selected from the group consisting of 89, 53, 84, 33, 92, 95, 54, 58, 46, 82, 36, 10, 62, 47, 66, 32, 55, 53, and 13; wherein the change in susceptibility in step (c) is a decrease in susceptibility  
15 to saquinavir.

In another embodiment of this invention, the above method is provided of assessing the effectiveness of protease antiretroviral therapy, having a mutation at codon 82 and  
20 a secondary mutation at codon 61, or a mutation at codon 90 and secondary mutations at codons 74, 15, or 69, wherein the change in susceptibility in step (c) is an increase in susceptibility to saquinavir.

25 In another embodiment of this invention, a resistance test vector is provided comprising an HIV patient-derived segment comprising nucleic acid encoding protease having a mutation at codon 82 and secondary mutations at codons selected from the group consisting of 73, 55, 48, 20, 43,  
30 53, 90, 13, 84, 23, 33, 74, 32, 39, 60, 36, and 35, or a mutation at codon 90 and secondary mutations at codons selected from the group consisting of 53, 95, 54, 84, 82, 46, 13, 74, 55, 85, 20, 72, 62, 66, 84, 48, 33, 73, 71,

64, 93, 23, 58, and 36 and an indicator gene, wherein the expression of the indicator gene is dependent upon the patient-derived segment.

5 In another embodiment of this invention, the above resistance test vector is provided, wherein the mutation of the patient derived segment at codon 82 is a substitution of alanine (A), phenylalanine (F), serine (S), or threonine (T) for valine(V) and the mutation at  
10 codon 90 is a substitution of methionine (M) for leucine (L).

**Phenotypic Susceptibility:**

Phenotypic assays provide information relating to drug  
15 resistance in the form of a fold-change in IC50 value, i.e. the ratio of the IC50 for the patient virus to that of a drug sensitive reference control. The significance of the fold change value with respect to treatment choices is limited by at least two factors: the reproducibility of  
20 the assay, and the achievable drug concentration at the site of action in the patient. For the PhenoSense™ assay described herein, the reproducibility cut-off is 2.5-fold. For most protease inhibitors, the level of reduction in susceptibility required to overcome the achievable plasma  
25 drug concentration is not well defined. However retrospective clinical studies using the 2.5-fold cutoff have suggested that this value is useful for predicting response to protease inhibitors, at least when used alone or in combination with reverse transcriptase inhibitors.  
30 Recently, the use of dual protease inhibitor based regimens (typically involving co-dosing of an inhibitor with ritonavir or nelfinavir) has become popular, since the plasma drug levels can be significantly boosted due to

inhibition of metabolic pathways. In cases such as these, it is likely that the clinically relevant fold-change cutoff will be higher, perhaps 10-fold. Future clinical studies will be required in order to accurately determine the actual clinical cutoff value.

As used herein, what it is understood to mean "secondary mutations" in addition to the discussion on pages 7 and 8 of this specification, is that other mutations, not currently recognized as resistance-associated, may also be defined as "secondary mutations" if they enhance the effects of primary mutations.

#### **Example 17**

#### **Predicting Response to Protease Inhibitors by Characterization of Amino Acid 82 of HIV-1 Protease.**

In one embodiment of this invention, changes in the amino acid at position 82 of the protease protein of HIV-1 are evaluated using the following method comprising: (i) collecting a biological sample from an HIV-1 infected subject; (ii) evaluating whether the biological sample contains nucleic acid encoding HIV-1 protease having a valine to alanine (V82A), phenylalanine (V82F), serine (V82S), threonine (V82T), or other amino acid substitution at codon 82 ("V82 mutations"); and (iii) determining susceptibility to protease inhibitors (PRI).

The biological sample comprises whole blood, blood components including peripheral mononuclear cells (PBMC), serum, plasma (prepared using various anticoagulants such as EDTA, acid citrate-dextrose, heparin), tissue biopsies, cerebral spinal fluid (CSF), or other cell, tissue or body

fluids. In another embodiment, the HIV-1 nucleic acid (genomic RNA) or reverse transcriptase protein can be isolated directly from the biological sample or after purification of virus particles from the biological sample. Evaluating whether the amino acid at position 82 of the HIV-1 protease is mutated to alanine, phenylalanine, serine, threonine, or other amino acids, can be performed using various methods, such as direct characterization of the viral nucleic acid encoding protease or direct characterization of the protease protein itself. Defining the amino acid at position 82 of protease can be performed by direct characterization of the protease protein by conventional or novel amino acid sequencing methodologies, epitope recognition by antibodies or other specific binding proteins or compounds. Alternatively, the amino acid at position 82 of the HIV-1 protease protein can be defined by characterizing amplified copies of HIV-1 nucleic acid encoding the protease protein. Amplification of the HIV-1 nucleic acid can be performed using a variety of methodologies including reverse transcription-polymerase chain reaction (RT-PCR), NASBA, SDA, RCR, or 3SR. The nucleic acid sequence encoding HIV protease at codon 82 can be determined by direct nucleic acid sequencing using various primer extension-chain termination (Sanger, ABI/PE and Visible Genetics) or chain cleavage (Maxam and Gilbert) methodologies or more recently developed sequencing methods such as matrix assisted laser desorption-ionization time of flight (MALDI-TOF) or mass spectrometry (Sequenom, Gene Trace Systems). Alternatively, the nucleic acid sequence encoding amino acid position 82 can be evaluated using a variety of probe hybridization methodologies, such as genechip



hybridization sequencing (Affymetrix), line probe assay (LiPA; Murex), and differential hybridization (Chiron).

In a preferred embodiment of this invention, evaluation of protease inhibitor susceptibility and of whether amino acid position 82 of HIV-1 protease was wild type or mutant was carried out using a phenotypic susceptibility assay or genotypic assay, respectively, using resistance test vector DNA prepared from the biological sample. In one embodiment, plasma sample was collected, viral RNA was purified and an RT-PCR methodology was used to amplify a patient derived segment encoding the HIV-1 protease and reverse transcriptase regions. The amplified patient derived segments were then incorporated, via DNA ligation and bacterial transformation, into an indicator gene viral vector thereby generating a resistance test vector. Resistance test vector DNA was isolated from the bacterial culture and the phenotypic susceptibility assay was carried out and analyzed as described in Example 1.

The nucleic acid (DNA) sequence of the patient derived HIV-1 protease and reverse transcriptase regions was determined using a fluorescence detection chain termination cycle sequencing methodology (ABI/PE). The method was used to determine a consensus nucleic acid sequence representing the combination of sequences of the mixture of HIV-1 variants existing in the subject sample (representing the quasispecies), and to determine the nucleic acid sequences of individual variants. Genotypes are analyzed as lists of amino acid differences between virus in the patient sample and a reference laboratory strain of HIV-1, NL4-3. Genotypes and corresponding phenotypes (fold-change in IC50 values) are entered in a



relational database linking these two results with patient information. Large datasets can then be assembled from patient virus samples sharing particular characteristics, such as the presence of any given mutation or reduced susceptibility to any drug or combination of drugs.

**(a) Protease inhibitor susceptibility of viruses containing mutations at amino acid 82 of HIV-1 Protease.**

Phenotypic susceptibility profiles of 270 patient virus samples that contained a mutation at position 82 (but not at positions 30 or 50, which are primary mutations associated with resistance to nelfinavir and amprenavir, respectively) were analyzed. According to most published guidelines, such viruses are expected to be resistant to ritonavir, nelfinavir, indinavir, and saquinavir. However, only 61.7% of these samples displayed reduced susceptibility to saquinavir using a 2.5-fold threshold (Table 13), while 31.2% and 40.0% displayed reduced susceptibility to saquinavir and indinavir, respectively, using a 10-fold threshold (Table 14). Thus, there was poor correlation between the presence of mutations at position 82 and saquinavir or indinavir susceptibility.

**(b) Indinavir susceptibility (fold change threshold 10) of viruses containing combinations of mutations at amino acid 82 and one secondary mutation in HIV-1 Protease.**

To explore the possibility that indinavir resistance (fold change in IC50 > 10-fold) in viruses containing mutations at position 82 requires the presence of other specific mutations, decreased indinavir susceptibility (fold-change in IC50 greater than 10) in viruses containing V82

mutations was correlated with the presence of mutations at other positions. This analysis revealed several positions (most strongly 73, 55, 48, 20, 43, 53, and 90) that decreased indinavir susceptibility significantly in combination with V82 mutations, compared to when these other mutations were absent (see Table 15). The presence of a mutation at position 13 significantly decreased the proportion of samples that had reduced indinavir susceptibility (45.9% vs. 62.2%; Table 15). In other words, the absence of a mutation at position 13 was correlated with decreased susceptibility to indinavir.

**(c) Saquinavir susceptibility (fold change threshold 2.5) of viruses containing combinations of mutations at amino acid 82 and one secondary mutation in HIV-1 Protease.**

To explore the possibility that saquinavir resistance in viruses containing mutations at position 82 requires the presence of other specific mutations, decreased saquinavir susceptibility (fold-change in IC50 greater than 2.5-fold) in viruses containing V82 mutations was correlated with the presence of mutations at other positions. This analysis revealed that several positions (most strongly 84, 48, 23, 73, 53, 33, 74, 20, and 90) were associated with reduced saquinavir susceptibility (See Table 16). The combination of mutations at position 82 with a mutation at position 32 or 39 significantly decreased the proportion of samples that had reduced saquinavir susceptibility (Table 16). In other words, the absence of a mutation at position 32 or 39 was correlated with decreased susceptibility to indinavir.

(d) Saquinavir susceptibility (fold change threshold 10) of viruses containing combinations of mutations at amino acid 82 and one secondary mutation in HIV-1 Protease.

5 To explore the possibility that saquinavir resistance in  
viruses containing mutations at position 82 requires the  
presence of other specific mutations, decreased saquinavir  
susceptibility (fold-change in IC50 greater than 10 fold)  
in viruses containing V82 mutations was correlated with  
10 the presence of mutations at other positions. This  
analysis revealed that several positions (most strongly  
48, 23, 84, 53, 74, 20, 60, 33, 36, 35, and 90) were  
associated with reduced saquinavir susceptibility (See  
Table 17). The combination of mutations at position 82  
15 with a mutation at position 32 or 46 significantly  
decreased the proportion of samples that had reduced  
saquinavir susceptibility (Table 17). In other words, the  
absence of a mutation at position 32 or 46 was correlated  
with decreased susceptibility to indinavir.

20

#### Example 18

**Predicting Response to Protease Inhibitors by  
Characterization of Amino Acid 90 of HIV-1 Protease.**

25 In one embodiment of this invention, changes in the amino  
acid at position 90 of the protease protein of HIV-1 are  
evaluated using the following method comprising: (i)  
collecting a biological sample from an HIV-1 infected  
subject; (ii) evaluating whether the biological sample  
30 contains nucleic acid encoding HIV-1 protease having a  
leucine to methionine (L90M) substitution at codon 90; and  
(iii) determining susceptibility to protease inhibitors  
(PRI).

The biological sample comprises whole blood, blood components including peripheral mononuclear cells (PBMC), serum, plasma (prepared using various anticoagulants such as EDTA, acid citrate-dextrose, heparin), tissue biopsies, cerebral spinal fluid (CSF), or other cell, tissue or body fluids. In another embodiment, the HIV-1 nucleic acid (genomic RNA) or reverse transcriptase protein can be isolated directly from the biological sample or after purification of virus particles from the biological sample. Evaluating whether the amino acid at position 90 of the HIV-1 protease is mutated to methionine, can be performed using various methods, such as direct characterization of the viral nucleic acid encoding protease or direct characterization of the protease protein itself. Defining the amino acid at position 90 of protease can be performed by direct characterization of the protease protein by conventional or novel amino acid sequencing methodologies, epitope recognition by antibodies or other specific binding proteins or compounds. Alternatively, the amino acid at position 90 of the HIV-1 protease protein can be defined by characterizing amplified copies of HIV-1 nucleic acid encoding the protease protein. Amplification of the HIV-1 nucleic acid can be performed using a variety of methodologies including reverse transcription-polymerase chain reaction (RT-PCR), NASBA, SDA, RCR, or 3SR. The nucleic acid sequence encoding HIV protease at codon 90 can be determined by direct nucleic acid sequencing using various primer extension-chain termination (Sanger, ABI/PE and Visible Genetics) or chain cleavage (Maxam and Gilbert) methodologies or more recently developed sequencing methods such as matrix assisted laser desorption-ionization time of flight (MALDI-TOF) or mass

spectrometry (Sequenom, Gene Trace Systems). Alternatively, the nucleic acid sequence encoding amino acid position 90 can be evaluated using a variety of probe hybridization methodologies, such as genechip hybridization sequencing (Affymetrix), line probe assay (LiPA; Murex), and differential hybridization (Chiron).

In a preferred embodiment of this invention, evaluation of protease inhibitor susceptibility and of whether amino acid position 90 of HIV-1 protease was wild type or methionine, was carried out using a phenotypic susceptibility assay or genotypic assay, respectively, using resistance test vector DNA prepared from the biological sample. In one embodiment, plasma sample was collected, viral RNA was purified and an RT-PCR methodology was used to amplify a patient derived segment encoding the HIV-1 protease and reverse transcriptase regions. The amplified patient derived segments were then incorporated, via DNA ligation and bacterial transformation, into an indicator gene viral vector thereby generating a resistance test vector. Resistance test vector DNA was isolated from the bacterial culture and the phenotypic susceptibility assay was carried out and analyzed as described in Example 1. The nucleic acid (DNA) sequence of the patient derived HIV-1 protease and reverse transcriptase regions was determined using a fluorescence detection chain termination cycle sequencing methodology (ABI/PE). The method was used to determine a consensus nucleic acid sequence representing the combination of sequences of the mixture of HIV-1 variants existing in the subject sample (representing the quasispecies), and to determine the nucleic acid sequences of individual variants. Genotypes are analyzed as lists of

amino acid differences between virus in the patient sample and a reference laboratory strain of HIV-1, NL4-3. Genotypes and corresponding phenotypes (fold-change in IC50 values) are entered in a relational database linking these two results with patient information. Large datasets can then be assembled from patient virus samples sharing particular characteristics, such as the presence of any given mutation or reduced susceptibility to any drug or combination of drugs.

**(a) Protease inhibitor susceptibility of viruses containing mutations at amino acid 90 of HIV-1 Protease.**

Phenotypic susceptibility profiles of 333 patient virus samples which contained a mutation at position 90 (L90M) but not at positions 30 or 50, which are primary mutations associated with resistance to nelfinavir and amprenavir, respectively) were analyzed. According to most published guidelines, such viruses are expected to be resistant to ritonavir, nelfinavir, indinavir, and saquinavir. However, only 79.3% and 84.7% of these samples displayed reduced susceptibility to saquinavir and indinavir, respectively, using a 2.5-fold threshold (Table 13), while 43.5% and 53.8% displayed reduced susceptibility to saquinavir and indinavir, respectively, using a 10-fold threshold (Table 14). Thus, there was poor correlation between the presence of mutations at position 90 and saquinavir or indinavir susceptibility.

**(b) Indinavir susceptibility (fold change threshold 2.5) of viruses containing combinations of mutations at amino acid 90 and one secondary mutation in HIV-1 Protease.**

5 To explore the possibility that indinavir resistance in  
viruses containing a mutation at position 90 requires the  
presence of other specific mutations, decreased indinavir  
susceptibility (fold-change in IC50 greater than 2.5) in  
10 viruses containing L90M was correlated with the presence  
of mutations at other positions. This analysis revealed  
several other positions (most strongly 53, 95, 54, 84, 82  
and 46) that decreased indinavir susceptibility  
significantly in combination with the L90M mutation,  
compared to when these other mutations were absent (see  
15 Table 18). The presence of a mutation at position 13 or 74  
significantly decreased the proportion of samples that had  
reduced indinavir susceptibility (Table 18). In other  
words, the absence of mutations at position 13 or 74 was  
correlated with decreased susceptibility to indinavir.

20

**(c) Indinavir susceptibility (fold change threshold 10) of viruses containing combinations of mutations at amino acid 90 and one secondary mutation in HIV-1 Protease.**

25 To explore the possibility that indinavir resistance in  
viruses containing a mutation at position 90 requires the  
presence of other specific mutations, decreased indinavir  
susceptibility (fold-change in IC50 greater than 10) in  
viruses containing L90M was correlated with the presence  
30 of mutations at other positions. This analysis revealed  
several secondary positions (most strongly 95, 55, 54, 82,  
85, 84, 20, 72, and 62) that decreased indinavir  
susceptibility significantly in combination with the L90M



mutation, compared to when these other mutations were absent (see Table 19). The presence of a mutation at position 74 significantly decreased the proportion of samples that had reduced indinavir susceptibility (27.5% vs. 57.3%; Table 19). In other words, the absence of a mutation at position 74 was correlated with decreased susceptibility to indinavir.

**(d) Saquinavir susceptibility (fold change threshold 2.5) of viruses containing combinations of mutations at amino acid 90 and one secondary mutation in HIV-1 Protease.**

To explore the possibility that saquinavir resistance in viruses containing a mutation at position 90 requires the presence of other specific mutations, decreased saquinavir susceptibility (fold-change in IC50 greater than 2.5) in viruses containing L90M was correlated with the presence of mutations at other positions. This analysis revealed several other positions (most strongly 53, 66, 84, 54, 48, 33, 73, 20, and 71) that decreased saquinavir susceptibility significantly in combination with the L90M mutation, compared to when these other mutations were absent (see Table 20). The presence of a mutation at position 64 or 93 significantly decreased the proportion of samples that had reduced saquinavir susceptibility (Table 20). In other words, the absence of a mutation at position 64 or 93 was correlated with decreased susceptibility to saquinavir.



**(e) Saquinavir susceptibility (fold change threshold 10) of viruses containing combinations of mutations at amino acid 90 and one secondary mutation in HIV-1 Protease.**

5 To explore the possibility that saquinavir resistance in  
viruses containing a mutation at position 90 requires the  
presence of other specific mutations, decreased saquinavir  
susceptibility (fold-change in IC50 greater than 10) in  
10 viruses containing L90M was correlated with the presence  
of mutations at other positions. This analysis revealed  
several other positions (most strongly 84, 53, 48, 23, 58,  
20, 36, and 54) that decreased saquinavir susceptibility  
significantly in combination with the L90M mutation,  
compared to when these other mutations were absent (see  
15 Table 21). The presence of a mutation at position 64, 77  
or 93 significantly decreased the proportion of samples  
that had reduced saquinavir susceptibility (Table 21). In  
other words, the absence of a mutation at position 64, 77  
or 93 was correlated with decreased susceptibility to  
20 saquinavir.

**(f) Saquinavir susceptibility (fold change threshold 2.5) of viruses containing combinations of mutations at amino acid 90 and many secondary mutations in HIV-1 Protease.**

25 To explore the possibility that saquinavir resistance in  
viruses containing mutations at position 90 requires the  
presence of some defined number of other mutations,  
decreased saquinavir susceptibility (fold-change in IC50  
30 greater than 2.5) in viruses containing L90M was  
correlated with the number of mutations at secondary  
positions. The following positions were considered: 10,  
20, 52, 53, 54, 66, 71, 73, and 84; positions 53 and 84

were weighted twice, yielding a saquinavir resistance-associated mutation count. This analysis revealed that 100% of samples with L90M and a mutation count of at least 5 had reduced saquinavir susceptibility (See Table 22). Combination with 3 or 4 other secondary mutations also significantly increased the proportion of samples that had reduced saquinavir susceptibility (85.7% and 97.3%, respectively; see Table 22).

**Table 13. PRI Susceptibility of Viruses with Mutations at 82 and/or 90 (fold change threshold > 2.5).**

| Drug       | Percent of viruses with indicated primary mutation(s) with reduced susceptibility (fold change in IC <sub>50</sub> > 2.5) |      |
|------------|---|------|
|            | V82 mutations   | L90M |
| Amprenavir | 60.0  | 60.4 |
| Indinavir  | 92.2  | 84.7 |
| Nelfinavir | 94.4  | 97.0 |
| Ritonavir  | 97.8  | 93.4 |
| Saquinavir | 61.7  | 79.3 |

**Table 14. PRI Susceptibility of Viruses with Mutations at 82 and/or 90 (fold change threshold > 10).**

| Drug       | Percent of viruses with indicated primary mutation(s) with reduced susceptibility (fold change in IC <sub>50</sub> > 10) |      |
|------------|--|------|
|            | V82 mutations  | L90M |
| Amprenavir | 10.4   | 12.9 |
| Indinavir  | 60.0   | 53.8 |
| Nelfinavir | 68.9   | 74.5 |
| Ritonavir  | 89.3   | 66.1 |
| Saquinavir | 31.2   | 43.5 |

**Table 15. Correlation Between V82 mutations, Secondary Mutations, and Indinavir Susceptibility (fold change threshold > 10).**

|    | position | + | or - | n   | mt % >10 | wt % >10 | p value |
|----|----------|---|------|-----|----------|----------|---------|
| 5  | 73       | + |      | 22  | 90.9     | 57.3     | 0.0011  |
|    | 55       | + |      | 25  | 80.0     | 58.0     | 0.0238  |
|    | 48       | + |      | 35  | 77.1     | 57.4     | 0.0188  |
|    | 20       | + |      | 77  | 76.6     | 53.4     | <0.001  |
| 10 | 43       | + |      | 34  | 76.5     | 57.6     | 0.0258  |
|    | 53       | + |      | 33  | 75.8     | 57.8     | 0.0349  |
|    | 90       | + |      | 135 | 74.1     | 45.9     | <0.001  |
|    | 72       | + |      | 56  | 73.2     | 56.5     | 0.0160  |
|    | 35       | + |      | 91  | 72.5     | 53.6     | 0.0019  |
| 15 | 54       | + |      | 188 | 71.3     | 34.1     | <0.001  |
|    | 71       | + |      | 184 | 70.7     | 37.2     | <0.001  |
|    | 36       | + |      | 99  | 69.7     | 54.4     | 0.0091  |
|    | 10       | + |      | 224 | 66.1     | 30.4     | <0.001  |
|    | 82       |   |      | 270 | 60.0     |          |         |
| 20 | 13       | - |      | 37  | 45.9     | 62.2     | 0.0458  |

**Table 16. Correlation Between V82 mutations, Secondary Mutations, and Saquinavir Susceptibility (fold change threshold > 2.5)**

|    | position | + | or - | n   | mt % >2.5 | wt % >2.5 | p value |
|----|----------|---|------|-----|-----------|-----------|---------|
| 25 | 84       | + |      | 36  | 100.0     | 55.8      | <0.001  |
|    | 48       | + |      | 35  | 97.1      | 56.4      | <0.001  |
|    | 23       | + |      | 11  | 90.9      | 60.5      | 0.0358  |
| 30 | 73       | + |      | 22  | 90.9      | 59.1      | 0.0018  |
|    | 53       | + |      | 33  | 87.9      | 58.1      | <0.001  |
|    | 33       | + |      | 24  | 87.5      | 59.2      | 0.0041  |
|    | 74       | + |      | 25  | 84.0      | 59.4      | 0.0113  |
|    | 20       | + |      | 77  | 83.1      | 53.1      | <0.001  |
| 35 | 90       | + |      | 135 | 82.2      | 41.0      | <0.001  |
|    | 43       | + |      | 34  | 79.4      | 59.1      | 0.0162  |
|    | 36       | + |      | 99  | 75.8      | 53.5      | <0.001  |
|    | 41       | + |      | 79  | 74.7      | 56.3      | 0.0032  |
|    | 54       | + |      | 187 | 74.3      | 32.9      | <0.001  |
| 40 | 71       | + |      | 183 | 74.3      | 34.9      | <0.001  |
|    | 35       | + |      | 91  | 73.6      | 55.6      | 0.0028  |
|    | 10       | + |      | 223 | 69.5      | 23.9      | <0.001  |
|    | 82       |   |      | 269 | 61.7      |           |         |
|    | 32       | - |      | 24  | 37.5      | 64.1      | 0.0106  |
| 45 | 39       | - |      | 4   | 0.0       | 62.6      | 0.0207  |

**Table 17. Correlation Between V82 mutations, Secondary Mutations, and Saquinavir Susceptibility (fold change threshold > 10)**

|    | position | + or - | n   | mt % >10 | wt %>10 | p value |
|----|----------|--------|-----|----------|---------|---------|
| 5  | 48       | +      | 35  | 82.9     | 23.5    | <0.001  |
|    | 23       | +      | 11  | 81.8     | 29.1    | <0.001  |
|    | 84       | +      | 36  | 72.2     | 24.9    | <0.001  |
|    | 53       | +      | 33  | 69.7     | 25.8    | <0.001  |
| 10 | 74       | +      | 25  | 56.0     | 28.7    | 0.0062  |
|    | 20       | +      | 77  | 55.8     | 21.4    | <0.001  |
|    | 60       | +      | 30  | 50.0     | 28.9    | 0.0181  |
|    | 33       | +      | 24  | 50.0     | 29.4    | 0.0352  |
|    | 36       | +      | 99  | 47.5     | 21.8    | <0.001  |
| 15 | 35       | +      | 91  | 44.0     | 24.7    | 0.0011  |
|    | 90       | +      | 135 | 43.0     | 19.4    | <0.001  |
|    | 41       | +      | 79  | 41.8     | 26.8    | 0.0126  |
|    | 62       | +      | 119 | 41.2     | 23.3    | 0.0013  |
|    | 54       | +      | 187 | 39.0     | 13.4    | <0.001  |
| 20 | 71       | +      | 183 | 37.7     | 17.4    | <0.001  |
|    | 10       | +      | 223 | 35.0     | 13.0    | 0.0019  |
|    | 82       |        | 269 | 31.2     |         |         |
|    | 46       | -      | 156 | 26.3     | 38.1    | 0.0275  |
|    | 32       | -      | 24  | 12.5     | 33.1    | 0.0268  |
| 25 |          |        |     |          |         |         |

**Table 18. Correlation Between L90M, Secondary Mutations, and Indinavir Susceptibility (fold change threshold > 2.5) .**

|    | position | + or - | n   | mt % >2.5 | wt %>2.5 | p value |
|----|----------|--------|-----|-----------|----------|---------|
| 30 | 53       | +      | 29  | 100.0     | 83.2     | 0.0064  |
|    | 95       | +      | 23  | 100.0     | 83.5     | 0.0189  |
|    | 54       | +      | 129 | 98.4      | 76.0     | <0.001  |
| 35 | 84       | +      | 104 | 97.1      | 79.0     | <0.001  |
|    | 82       | +      | 135 | 94.1      | 78.3     | <0.001  |
|    | 46       | +      | 164 | 93.3      | 76.3     | <0.001  |
|    | 73       | +      | 117 | 92.3      | 80.6     | 0.0027  |
|    | 71       | +      | 233 | 91.4      | 69.0     | <0.001  |
| 40 | 20       | +      | 115 | 91.3      | 81.2     | 0.0095  |
|    | 10       | +      | 255 | 90.2      | 66.7     | <0.001  |
|    | 63       | +      | 325 | 85.5      | 50.0     | 0.0214  |
|    | 90       |        | 333 | 84.7      |          |         |
|    | 13       | -      | 77  | 76.6      | 87.1     | 0.0226  |
| 45 | 74       | -      | 40  | 67.5      | 87.0     | 0.0028  |

**Table 19. Correlation Between L90M, Secondary Mutations, and Indinavir Susceptibility (fold change threshold > 10).**

|    | position | + | or - | n   | mt % >10 | wt % >10 | p value |
|----|----------|---|------|-----|----------|----------|---------|
| 5  | 95       | + |      | 23  | 82.6     | 51.6     | 0.0030  |
|    | 55       | + |      | 22  | 81.8     | 51.8     | 0.0048  |
|    | 54       | + |      | 129 | 81.4     | 36.3     | <0.001  |
|    | 82       | + |      | 135 | 74.1     | 39.9     | <0.001  |
|    | 85       | + |      | 23  | 73.9     | 52.3     | 0.0346  |
| 10 | 84       | + |      | 104 | 70.2     | 46.3     | <0.001  |
|    | 20       | + |      | 115 | 66.1     | 47.2     | 0.0103  |
|    | 72       | + |      | 87  | 64.4     | 50.0     | 0.0141  |
|    | 62       | + |      | 154 | 63.6     | 45.3     | <0.001  |
|    | 46       | + |      | 164 | 63.4     | 44.4     | <0.001  |
| 15 | 36       | + |      | 114 | 63.2     | 48.9     | 0.0088  |
|    | 10       | + |      | 255 | 63.1     | 23.1     | <0.001  |
|    | 71       | + |      | 233 | 60.9     | 37.0     | <0.001  |
|    | 90       |   |      | 333 | 53.8     |          |         |
|    | 74       | - |      | 40  | 27.5     | 57.3     | <0.001  |

**Table 20. Correlation Between L90M, Secondary Mutations, and Saquinavir Susceptibility (fold change threshold > 2.5).**

|    | position | + | or - | n   | mt % >2.5 | wt % >2.5 | p value |
|----|----------|---|------|-----|-----------|-----------|---------|
| 25 | 53       | + |      | 29  | 100.0     | 77.3      | <0.001  |
|    | 66       | + |      | 13  | 100.0     | 78.4      | 0.0459  |
|    | 84       | + |      | 104 | 98.1      | 70.7      | <0.001  |
| 30 | 54       | + |      | 129 | 96.9      | 68.1      | <0.001  |
|    | 48       | + |      | 22  | 95.5      | 78.1      | 0.0362  |
|    | 33       | + |      | 37  | 94.6      | 77.4      | 0.0076  |
|    | 73       | + |      | 117 | 89.7      | 73.6      | <0.001  |
|    | 20       | + |      | 115 | 89.6      | 73.9      | <0.001  |
| 35 | 71       | + |      | 233 | 88.4      | 58.0      | <0.001  |
|    | 36       | + |      | 114 | 87.7      | 74.9      | 0.0038  |
|    | 10       | + |      | 255 | 86.3      | 56.4      | <0.001  |
|    | 37       | + |      | 104 | 85.6      | 76.4      | 0.0365  |
|    | 63       | + |      | 325 | 80.3      | 37.5      | 0.0112  |
| 40 | 90       |   |      | 333 | 79.3      |           |         |
|    | 93       | - |      | 187 | 74.3      | 85.6      | 0.0080  |
|    | 64       | - |      | 66  | 63.6      | 83.1      | <0.001  |

**Table 21. Correlation Between L90M, Secondary Mutations, and Saquinavir Susceptibility (fold change threshold > 10) .**

|    | position | + or - | n   | mt % >10 | wt % >10 | p value |
|----|----------|--------|-----|----------|----------|---------|
| 5  | 84       | +      | 104 | 84.6     | 24.9     | <0.001  |
|    | 53       | +      | 29  | 82.8     | 39.8     | <0.001  |
|    | 48       | +      | 22  | 81.8     | 40.8     | <0.001  |
|    | 23       | +      | 12  | 75.0     | 42.4     | 0.0260  |
| 10 | 58       | +      | 30  | 63.3     | 41.6     | 0.0182  |
|    | 20       | +      | 115 | 61.7     | 33.9     | <0.001  |
|    | 36       | +      | 114 | 61.4     | 34.2     | <0.001  |
|    | 54       | +      | 129 | 60.5     | 32.8     | <0.001  |
|    | 35       | +      | 109 | 53.2     | 38.8     | 0.0091  |
| 15 | 73       | +      | 117 | 51.3     | 39.4     | 0.0240  |
|    | 10       | +      | 255 | 50.6     | 20.5     | <0.001  |
|    | 71       | +      | 233 | 49.8     | 29.0     | <0.001  |
|    | 62       | +      | 154 | 49.4     | 38.5     | 0.0306  |
|    | 90       |        | 333 | 43.5     |          |         |
| 20 | 93       | -      | 187 | 38.0     | 50.7     | 0.0135  |
|    | 77       | -      | 139 | 35.3     | 49.5     | 0.0066  |
|    | 64       | -      | 66  | 33.3     | 46.1     | 0.0409  |

**Table 22. Association Between Saquinavir Susceptibility, L90M, and Number of Resistance Associated Mutations.**

|    | Number of secondary mutations | n  | % with SQV FC > 2.5 | Mean SQV fold change |
|----|-------------------------------|----|---------------------|----------------------|
|    | 0                             | 17 | 23.5                | 2.4                  |
| 30 | 1                             | 40 | 25.0                | 2.4                  |
|    | 2                             | 49 | 69.4                | 5.4                  |
|    | 3                             | 63 | 85.7                | 10.0                 |
|    | 4                             | 74 | 97.3                | 36.6                 |
|    | 5                             | 34 | 100                 | 50.3                 |
| 35 | 6 or more                     | 56 | 100                 | 94.2                 |

Tables 23-27 show results as indicated using the above procedures as described in Examples 17 and 18.

5 **Table 23. Correlation Between L90M, Secondary Mutations, and Amprenavir Susceptibility (fold change threshold > 2.5) .**

| Amprenavir |          |   |      |     |           |          |         |
|------------|----------|---|------|-----|-----------|----------|---------|
|            | position | + | or - | n   | mt % >2.5 | wt %>2.5 | p value |
| 10         | 89       | + |      | 11  | 90.9      | 59.3     | 0.0298  |
|            | 53       | + |      | 29  | 89.7      | 57.6     | <0.001  |
|            | 84       | + |      | 104 | 86.5      | 48.5     | <0.001  |
|            | 33       | + |      | 37  | 83.8      | 57.4     | 0.0012  |
| 15         | 92       | + |      | 24  | 83.3      | 58.6     | 0.0120  |
|            | 95       | + |      | 23  | 82.6      | 58.7     | 0.0174  |
|            | 54       | + |      | 129 | 80.6      | 47.5     | <0.001  |
|            | 58       | + |      | 30  | 76.7      | 58.7     | 0.0400  |
| 20         | 46       | + |      | 164 | 75.0      | 46.2     | <0.001  |
|            | 82       | + |      | 135 | 70.4      | 53.5     | 0.0014  |
|            | 36       | + |      | 114 | 70.2      | 55.3     | 0.0055  |
|            | 10       | + |      | 255 | 69.4      | 30.8     | <0.001  |
| 25         | 62       | + |      | 154 | 66.2      | 55.3     | 0.0272  |
|            | 90       |   |      | 333 | 60.4      |          |         |
|            | 74       | - |      | 40  | 45.0      | 62.5     | 0.0269  |
|            | 15       | - |      | 53  | 43.4      | 63.6     | 0.0050  |

30 **Table 24. Correlation Between L90M, Secondary Mutations, and Amprenavir Susceptibility (fold change threshold > 10) .**

35

| Amprenavir |          |        |     |       |         |         |
|------------|----------|--------|-----|-------|---------|---------|
|            | position | + or - | n   | % >10 | wt %>10 | p value |
| 40         | 47       | +      | 5   | 80.0  | 11.9    | 0.0011  |
|            | 33       | +      | 37  | 48.6  | 8.4     | <0.001  |
|            | 66       | +      | 13  | 38.5  | 11.9    | 0.0166  |
|            | 32       | +      | 16  | 37.5  | 11.7    | 0.0097  |
|            | 55       | +      | 22  | 31.8  | 11.6    | 0.0140  |
|            | 53       | +      | 29  | 27.6  | 11.5    | 0.0213  |
|            | 54       | +      | 129 | 24.0  | 5.9     | <0.001  |
|            | 84       | +      | 104 | 22.1  | 8.7     | 0.0010  |
| 45         | 13       | +      | 77  | 19.5  | 10.9    | 0.0424  |
|            | 46       | +      | 164 | 17.7  | 8.3     | 0.0080  |
|            | 10       | +      | 255 | 16.1  | 2.6     | <0.001  |
|            | 90       |        | 333 | 12.9  |         |         |
|            | 69       | -      | 37  | 2.7   | 14.2    | 0.0316  |

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**Table 25. Correlation Between V82, Secondary Mutations, and Indinavir Susceptibility (fold change threshold > 2.5)**

| Indinavir |          |   |      |     |           |          |         |
|-----------|----------|---|------|-----|-----------|----------|---------|
|           | position | + | or - | n   | mt % >2.5 | wt %>2.5 | p value |
| 5         | 84       | + |      | 37  | 100.0     | 91.0     | 0.0397  |
|           | 20       | + |      | 77  | 98.7      | 89.6     | 0.0064  |
|           | 72       | + |      | 56  | 98.2      | 90.7     | 0.0432  |
|           | 54       | + |      | 188 | 97.3      | 80.5     | <0.001  |
| 10        | 71       | + |      | 184 | 97.3      | 81.4     | <0.001  |
|           | 46       | + |      | 157 | 95.5      | 87.6     | 0.0155  |
|           | 93       | + |      | 133 | 95.5      | 89.1     | 0.0391  |
|           | 10       | + |      | 224 | 94.6      | 80.4     | 0.0034  |
| 15        | 82       |   |      | 270 | 92.2      |          |         |
|           | 37       | - |      | 108 | 88.0      | 95.1     | 0.0297  |
|           | 64       | - |      | 56  | 85.7      | 93.9     | 0.0451  |
|           | 13       | - |      | 37  | 70.3      | 95.7     | <0.001  |
|           | 45       | - |      | 12  | 58.3      | 93.8     | <0.001  |
|           |          |   |      |     |           |          |         |

**Table 26. Correlation Between V82, Secondary Mutations, and Amprenavir Susceptibility (fold change threshold > 2.5)**

| Amprenavir |          |   |      |     |           |          |         |
|------------|----------|---|------|-----|-----------|----------|---------|
|            | position | + | or - | n   | mt % >2.5 | wt %>2.5 | p value |
| 30         | 33       | + |      | 24  | 95.8      | 56.5     | <0.001  |
|            | 23       | + |      | 12  | 91.7      | 58.5     | 0.0176  |
|            | 84       | + |      | 37  | 86.5      | 55.8     | <0.001  |
|            | 32       | + |      | 24  | 83.3      | 57.7     | 0.0104  |
|            | 53       | + |      | 33  | 81.8      | 57.0     | 0.0042  |
| 35         | 90       | + |      | 135 | 70.4      | 49.6     | <0.001  |
|            | 37       | + |      | 108 | 66.7      | 55.6     | 0.0442  |
|            | 71       | + |      | 184 | 66.3      | 46.5     | 0.0016  |
|            | 46       | + |      | 157 | 65.6      | 52.2     | 0.0184  |
|            | 10       | + |      | 224 | 65.2      | 34.8     | <0.001  |
| 40         | 54       | + |      | 188 | 63.8      | 51.2     | 0.0356  |
|            | 82       |   |      | 270 | 60.0      |          |         |
|            | 61       | - |      | 21  | 38.1      | 61.8     | 0.0297  |



**Table 27. Correlation Between V82, Secondary Mutations, and Amprenavir Susceptibility (fold change threshold > 10).**

|    | Amprenavir |        |     |          |          |         |
|----|------------|--------|-----|----------|----------|---------|
|    | position   | + or - | n   | mt % >10 | wt % >10 | p value |
| 5  | 33         | +      | 24  | 50.0     | 6.5      | <0.001  |
|    | 11         | +      | 8   | 37.5     | 9.5      | 0.0394  |
|    | 84         | +      | 37  | 35.1     | 6.4      | <0.001  |
| 10 | 32         | +      | 24  | 25.0     | 8.9      | 0.0258  |
|    | 60         | +      | 30  | 23.3     | 8.8      | 0.0229  |
|    | 53         | +      | 33  | 21.2     | 8.9      | 0.0382  |
|    | 90         | +      | 135 | 14.8     | 5.9      | 0.0133  |
|    | 46         | +      | 157 | 14.6     | 4.4      | 0.0046  |
| 15 | 71         | +      | 184 | 13.0     | 4.7      | 0.0243  |
|    | 10         | +      | 224 | 12.5     | 0.0      | 0.0039  |
|    | 82         |        | 270 | 10.4     |          |         |

20 In Tables 13-27, the first column lists the various codon positions for HIV-1 protease for the secondary mutations and the primary mutation at codon 82 or 90.

25 The second column represents a positive (+) or negative (-) correlation between the change in resistance from the number of wild-type reference samples to those samples having the secondary mutation.

30 The fourth column, designates "mt %", as the percentage of samples having the secondary mutation and showing the indicated fold resistance to the specified protease inhibitor, (i.e, > 10 fold or > 2.5 fold).

35 The fifth column, designates "wt %", as the percentage of wild-type reference samples showing the indicated fold resistance, (i.e, > 10 fold or or 2.5 fold) to the specified protease inhibitor.

40 The sixth column represents the statistical P value for a correlation.

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The following list of mutations represents, by example, secondary mutations from a database for selected patient samples used to establish the above data in Tables 13-27. The mutations listed show the wild type reference amino acid and the possible various mutations for the substituted amino acid at the designated codon position for HIV-1 protease.

5  
  
10 L10F/I/R/V, I13V, K20I/M/R/T/V  
L23I, V32I, L33F/I/V  
E35/D/N/G, M36I/L/T/V, N37C/D/E/G/H/S/T  
P39A/Q/S/T, R41K/W/S, K43R/T  
K45R, M46I/L/V, G48M/S/V  
15 S53L/Y, I54A/L/M/S/T/V, K55N/R  
Q58E, D60E, I62/V/M,  
L63A/C/D/S/H/I/N/P/Q/R/S/T/V/Y  
I64L/M/V, I66F/L/T/V, A71I/L/T/V  
I72A/E/K/L/M/R/T/V, G73A/C/S/T, T74A/K/P/S  
20 V77I/T, V82A/F/S/T, I84A/M/V, I85V  
L90M, I93L/M, C95F

**Detailed Description of the Invention** (continued)

In another embodiment, the present invention provides a method for determining whether an HIV virus obtained from a patient infected with HIV is resistant to IDV, LPV, NFV and RTV which comprises determining whether a mutation at position 30 from D to N exists in the HIV protease obtained from the patient, wherein the presence of the mutation indicates that the virus is resistant to IDV, LPV, NFV and RTV.

In another embodiment, the present invention provides a method for determining whether an HIV virus obtained from a patient infected with HIV is resistant to IDV, LPV, NFV or RTV which comprises determining whether the virus is resistant to any one of IDV, LPV, NFV or RTV, wherein a determination that the virus is resistant to any one of IDV, LPV, NFV or RTV is indicative of the virus being resistant to IDV, LPV, NFV and RTV.

In a further embodiment, the present invention provides a method for determining cross resistance of an HIV virus to RTV and SQV which comprises determining (i) whether position 30 of the HIV protease is D, and (ii) whether the virus is resistant to NFV, wherein a mutation from D to N at position 30 of HIV protease and resistance of the virus to NFV are indicative of cross resistance to IDV and SQV.

In yet another embodiment the invention provides a method for determining whether an HIV virus obtained from a patient infected with HIV is resistant to LPV and IND which comprises determining whether position 50 of the HIV protease of the virus is I or V, wherein the determination that position 50 is V is indicative of the virus being resistant to LPV and IND. These and other embodiments of the invention are shown and described with reference to the accompanying Figures 6-28 in view of the specification contained herein.